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The Adenosinetriphosphatase Activity of Myofibrils Isolated from Skeletal Muscle

By S. V. PERRY
Biochemical Laboratory, University of Cambridge

(*Received* 31 *July* 1950)

Muscle has long been known as a source of many well characterized enzymes and proteins, but information about the localization of these substances within the cell is obtained mainly by inference and other indirect means. Direct investigation of the structural interrelationships of such constituents of muscle, most of which have been intensively studied in the purified form, should reveal considerable information about the working of the cell in general and the contractile process in particular. Muscle does not lend itself to the fractionation techniques which have been applied with considerable success to liver and kidney because the cells are not easily broken up by mechanical means, and the cytoplasm is extremely viscous owing to a high degree of structural organization. In consequence the procedures hitherto employed to isolate organized components from the muscle cell have been drastic. Behrens (1932) isolated nuclei from calf-heart muscle by dehydrating the tissue with organic solvents, grinding and finally centrifuging the powder in a benzene-chloroform mixture. The method of Stoneburg (1939) for isolating muscle nuclei, employing citric acid treatment followed by pepsin digestion in 1% hydrochloric acid, is hardly less drastic.

The main cytoplasmic constituent, the myofibrils, can be isolated in quantity by fixing the muscle in formalin for several days and then breaking up the tissue mechanically. Such preparations are useful for examination in the electron microscope (Hall, Jakus & Schmitt, 1946; Draper & Hodge, 1949), but are of limited value for more precise biochemical investigation. By weakening the muscle cell with trypsin at 0° and pH 7 Schick & Hass (1949) have indicated a useful method for obtaining myofibrils in amounts sufficient for biochemical study. These authors (1949) have shown that such myofibrils shorten in the presence of adenosinetriphosphate (ATP), and that when isolated from skeletal and heart muscle their solubility properties are in general similar to those of actomyosin (Ashley, Hass & Schick, 1950).

From the results of investigations on the adenosinetriphosphatase (ATPase) activity of extracted myosin and actomyosin, Mommaerts & Seraidarian (1947) and Braverman & Morgulis (1948) conclude that with the concentration of calcium and magnesium known to exist in muscle, the myosin present would split off phosphate from ATP at a rate which is about one-hundredth of the rate at which phosphate appears when muscle is contracting. The work described here was undertaken to investigate the ATP-splitting properties of the isolated myofibril and to see if it showed any marked differences in behaviour to the presumed myofibrillar component, actomyosin. A modified method of preparation was used as it soon became apparent that at 0° low concentrations of trypsin have a considerable effect on the myofibril. Even traces of the enzyme can attack both the myofibril and extracted myosin without destroying the ATPase activity, yet the physical properties of the myosin are considerably changed, particularly with respect to its interaction with actin. Gergely (1950) has recently reported independently a similar finding with myosin. A preliminary account of this work has been communicated to the Biochemical Society (Perry, 1950*a*).

EXPERIMENTAL

Methods

Preparation of myofibrils. The method is a modification of that used by Schick & Hass (1949). A 2–3 g. portion of psoas muscle from a freshly killed rabbit is dissected free from other tissues, allowed to stand for 30 min. at room temperature and frozen on the stage of a freezing microtome. The frozen tissue is sectioned into slices 25 μ. thick, which are rapidly transferred into 50 ml. of a well stirred, cold 0·08 M-borate buffer, pH 7·1. It is important that the borate solution is kept as cold as possible without freezing, and that the muscle slices are transferred to it whilst they are still in the frozen state to prevent the myofibrils from going into thaw rigor (Szent-Györgyi, 1949; Perry, 1950*b*). This shortening depends on the concentration of ATP in the thawed muscle and takes place very slowly or not at all at 0°. The effect can be eliminated by allowing the muscle to go into rigor mortis before freezing and sectioning. To the suspension of muscle cells 25 mg. of purified collagenase is added, and the enzyme is allowed to act at 0° for 3–7 hr. This period varies with the collagenase preparation and must be determined by experiment. The suspension is diluted with 150 ml. of the borate buffer and homogenized for 20 sec. in a chilled Waring blender. At this stage a drop of the suspension stained with methylene blue should show that practically all the cells have broken up into myofibrils. The myofibrils are now washed three times with borate buffer by centrifugation at 1500 *g*, these and subsequent operations being carried out at 0°. The first supernatant is usually slightly turbid, but later supernatants are clear. Large particles, synaeresed myofibrils, and cell fragments are now removed by resuspending in 100 ml. and centrifuging for 1 min. at 400 *g*. The material

which settles is discarded and the suspension centrifuged down again at 1500 *g*, resuspended in 100 ml. of borate and centrifuged for 1 min. at 400 *g*. The turbid supernatant is a very slowly settling suspension of myofibrils which usually contains 1–2 mg. protein/ml. As shown in Fig. 1 the suspension is quite homogeneous; the myofibrils are about 1 μ. wide, and occur singly and sometimes in bundles of two or three.

For preparations with trypsin the psoas slices were suspended in 200 ml. of ice-cold borate and 4 mg. of crystalline trypsin added. When microscopic examination indicated that the cells would readily rupture, usually after about 30 min., they were homogenized and the preparation carried out as for the method using collagenase.

Myosin and actomyosin. Myosin was prepared by the method of Bailey (1942). Actomyosin was the myosin B of Banga & Szent-Györgyi (1942) and was purified by three precipitations as in the method for myosin.

Adenosinetriphosphate. The Ba salt of ATP was prepared by a method similar to that of Needham (1942), and which included a final precipitation of the Ba salt with 50% (v/v) ethanol at pH 3. It was converted to the Na salt as recommended by Bailey (1942).

Adenosinetriphosphatase activity. ATPase determinations were carried out in 0·1 M-borate buffer, pH 6·9, and 0·1 M glycine buffer usually pH 8·6. In addition to activators ($CaCl_2$, $MgCl_2$, and KCl), 0·3 ml. ATP (2·0 mg. 10 min. hydrolysable P/ml.), and myofibril suspension, glass-distilled water was added to each enzyme test sample to bring the volume up to 2 ml. in all cases. After incubation, usually 15 min. at 25°, the reaction was stopped by 1·5 ml. of 10% trichloroacetic acid.

Collagenase. The preparation, kindly donated by Dr E. Bidwell of the Wellcome Research Foundation, was active against collagen, gelatin, and reticulin, but did not attack elastin. The dried powder contained 11·6 *Q* units of collagenase/mg. (Bidwell & van Heyningen, 1948), an active hyaluronidase and a small amount of lecithinase.

Trypsin. This preparation was the crystalline enzyme containing 50% $MgSO_4$, as supplied by the Armour Company.

Viscosity determinations. Determinations of viscosity and inhibition of actomyosin formation were carried out as described by Bailey & Perry (1947).

RESULTS

Trypsin-prepared myofibrils. Some difficulty was experienced in minimizing the action of trypsin on the myofibrils themselves yet at the same time allowing the enzyme to act long enough so that these structures could be readily liberated. It was found to be difficult also to remove the last traces of trypsin by washing three to four times on the centrifuge, with the result that within 2 or 3 days the myofibrils lost their morphological features. After longer storage at 0° the milky suspensions became almost clear, yet the adenosinetriphosphatase activity of the whole preparation did not fall; indeed in the early stages an increase was usually observed. The myofibrillar adenosinetriphosphatase is remarkably stable to trypsin, e.g. no loss in activity could be demonstrated when a preparation was treated at pH 7·1 and 0° for 106 min. with 0·015 mg. trypsin/ml.

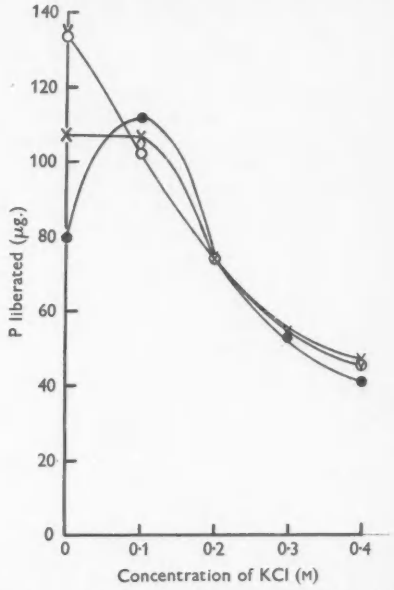

Fig. 2. The effect of KCl on the adenosinetriphosphatase activity of stored trypsin-prepared myofibrils. Enzymic activity measured with 0·005 M-$CaCl_2$ in glycine buffer, pH 8·6, at the following times after completion of the preparation: 1 hr., ●; 24 hr., ×; 48 hr., ○; 72 hr., v.

Table 1. *The adenosinetriphosphatase activity and soluble protein concentration of a trypsin-prepared myofibril suspension standing at* 0°

(Enzymic activity was measured in glycine buffer, pH 8·6, and 0·005 M-$CaCl_2$.)

Age of preparation (hr.)	Whole suspension: Protein concn. (mg./ml.)	Whole suspension: ATPase activity (μg. P/ml./15 min.)	Clear supernatant: Protein concn. (mg./ml.)	Clear supernatant: ATPase activity (μg. P/ml./15 min.)
1	1·51	243	0·12	Nil
24	1·51	275	0·50	Nil
48	1·51	280	0·65	29
72	1·51	277	0·83	64

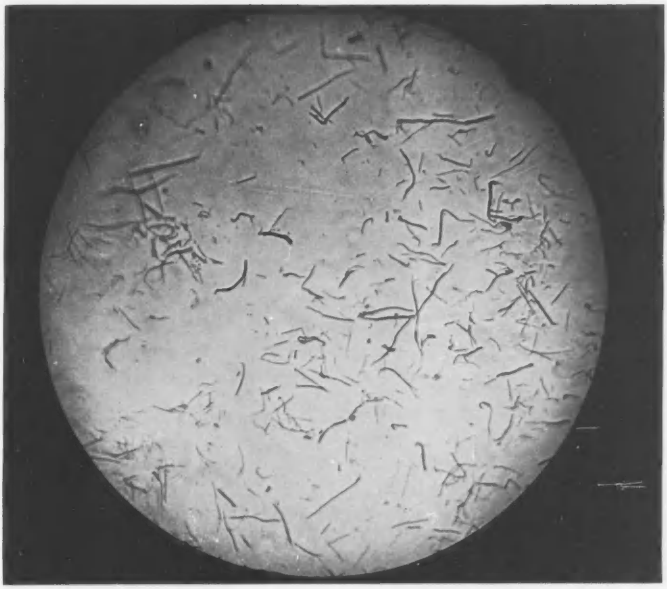

Fig. 1. Photomicrograph of collagenase-prepared myofibrils. Van Gieson stain. (× 280.)

Fig. 4. Electron micrograph of collagenase-prepared myofibril. Fixed by 5 min. exposure to formalin vapour before drying. Gold-palladium shadowed. (× 15,400.)

On storing suspensions of trypsin-prepared myofibrils in 0·08M-borate at 0°, within 2–3 days about one-third of the total nitrogen appeared in the clear supernatant liquid obtained by centrifugation and could be precipitated by trichloroacetic acid. The adenosinetriphosphatase activity of the whole increased. Ultimately, as the amount of soluble nitrogen increased, adenosinetriphosphatase activity passed into solution (Table 1) until, after a week or more, practically all the adenosinetriphosphatase was dissolved and could not be separated by centrifugation at 20,000 *g* for 30 min. Overnight dialysis against 0·05M-potassium chloride at pH 6·5–7·0 did not precipitate the enzyme, nor could any adenosinetriphosphatase activity be detected in the dialysate. Fig. 2 shows that freshly prepared myofibril suspensions have maximum adenosinetriphosphatase activity in 0·1M-potassium chloride when activated by 0·005M-calcium chloride in glycine buffer at pH 8·6. After the suspension has stood at 0° the effect of potassium chloride changes, and within a few days all concentrations inhibit the adenosinetriphosphatase. In parallel with this changing effect of potassium chloride, the enzymic activity in the absence of the salt increased.

During the period in which the adenosinetriphosphatase of the myofibrils was maintained, the fall in viscosity produced by addition of adenosinetriphosphate to a 0·6M-potassium chloride extract of the myofibrils diminished until finally it was almost absent, indicating that there was very little actomyosin in the extract.

Action of trypsin on myosin. It was considered that the changes in the properties of the myofibrils were due to the continued action of slight traces of trypsin. To confirm this, the action of the crystalline enzyme on myosin was studied. A dilute myosin gel (1–2 mg. protein/ml.) in 0·05M-potassium chloride, treated with crystalline trypsin (0·0045 mg./ml.) at 0°, became almost completely clear after a few hours. The adenosinetriphosphatase activity (measured in 0·1M-potassium chloride, 0·005M-calcium chloride, and 0·1M-glycine buffer, pH 8·6) increased by 20–30%; considerably greater increases were obtained in the absence of potassium chloride, but the ability to form actomyosin dropped appreciably, as shown in Fig. 3, and after some days became almost negligible although the myosin was still enzymically active. At pH 8·6 increasing concentrations of potassium chloride inhibited the calcium-activated adenosinetriphosphatase of trypsin-treated myosin in an identical fashion to their effect on stored trypsin-prepared myofibrils. The effect of progressive tryptic action on the viscosity of myosin is indicated in Table 2, which gives the figures for actomyosin viscosity in the presence of adenosinetriphosphate. As a constant amount of actin is added under comparable conditions in each case to the trypsin-treated myosin, the fall in viscosity of the dissociated actomyosin must indicate a fall in the viscosity of the myosin with progressive tryptic action.

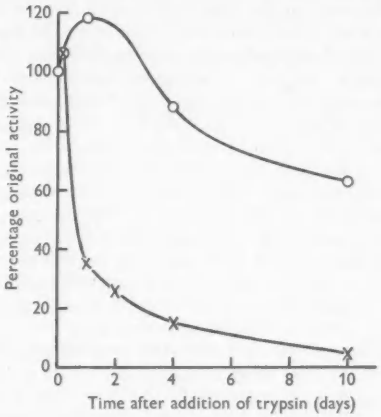

Fig. 3. The actomyosin-forming and the adenosinetriphosphatase activities of myosin treated with trypsin. Actomyosin-forming activity, ×; adenosinetriphosphatase activity, ○.

Table 2. *Effect of trypsin on the adenosinetriphosphatase and actomyosin-forming activities of myosin*

(The two centre columns show the specific viscosities at 0°, with and without 0·0004M-ATP, of the solutions obtained when samples of the trypsin-treated myosin were added to a constant amount of actin in each case. Enzymic activity was measured in glycine buffer, pH 8·6, containing 0·005M-$CaCl_2$.)

Time after addition of trypsin (hr.)	Specific viscosity of actomyosin		ATPase activity (μg. P/ml./15 min.)
	No ATP	ATP	
1·5	0·665	0·284	504
24	0·262	0·138	563
48	0·198	0·107	—
96	0·161	0·109	418
120	0·104	0·087	297
0 (control)	0·662	0·309	475

Collagenase-prepared myofibrils. Myofibrils prepared by this method show definite cross-striations which are visible without staining in the phase-contrast microscope, but are made more distinct with methylene blue. With van Gieson stain the A, I and Z bands can be very clearly distinguished. Electron microscope examination (Horne & Perry, 1951) reveals that the myofibrils have retained their fine structure (Fig. 4); filaments with the 400A. period are readily seen, and in general the picture is similar to that obtained by Draper & Hodge (1949) with myofibrils obtained from muscle fixed in

formalin. Examination of some fifteen preparations by this method indicated that they were quite homogeneous. The only recognizable contaminants seen were collagen-like filaments presumably of sarcolemmal origin, and quite infrequently very dense bodies considered from their size to be nuclei.

When 0·0004 M-adenosinetriphosphate and 0·002 M-magnesium chloride are added to the myofibrils suspended in borate buffer, the fibrils shorten and settle rapidly. Unlike threads made from extracted actomyosin which synearese isodimensionally under these conditions, myofibrils do not obviously get thinner, although the decrease in length may be considerable. Centrifugation at 1000 rev./min. for 20 sec. causes the adenosinetriphosphate-treated myofibrils to settle down in the tube, whereas the control myofibrils, treated with magnesium chloride only, require much longer to bring about the same effect. After prolonged centrifugation both settle down to approximately the same volume, suggesting that there is no great change in the water content.

Good preparations can be obtained from muscle which is allowed to go into rigor mortis by leaving overnight at room temperature. Like the normal preparations these myofibrils shorten in the presence of adenosinetriphosphate, whereas attempts to prepare myofibrils from muscle which had gone into thaw rigor gave rather granular, rapidly settling bodies, which were not obviously affected by adenosinetriphosphate.

The myofibrils keep their structure well in 0·08 M-borate. After storage at 0° for 1–2 weeks striations are still visible, little aggregation is to be seen and no adenosinetriphosphatase activity can be detected in the clear supernatant liquid obtained when the suspension is centrifuged.

Adenosinetriphosphatase activity of collagenase-prepared myofibrils. If the assumption is made that the protein concentration of a myofibril suspension is six times the nitrogen concentration, the usual adenosinetriphosphatase activity, Q_p, obtained at 25° with calcium activation ranges from 400–600 for collagenase-prepared myofibrils. (For definition of Q_p see Bailey, 1942.) It is unlikely that all the myofibrillar protein is myosin and preliminary determinations suggest that this protein makes up about 65–75 % of the total. Q_p values calculated on the basis of the amount of myosin present, and assuming a twofold increase in activity on raising the temperature from 25 to 37°, would compare quite favourably with those obtained by Bailey (1942) and Meyerhof & Polis (1947) for average Edsall myosin preparations. The slightly higher Q_p values obtained with the trypsin-prepared myofibrils are to be expected in the light of the results described earlier. It is of interest to note, in view of the emphasis in the literature given to the calcium-activated adenosinetriphosphatase of myosin, that Q_p values similar to those given with calcium can be obtained in the presence of low concentrations of magnesium in the absence of other salts.

Collagenase itself has no action on the calcium-activated adenosinetriphosphatase at pH 8·6, e.g. the adenosinetriphosphatase activity of collagenase-prepared myofibrils which had been treated for an additional 33 hr. with collagenase (5 hr. with 0·6 mg./ml. followed by 28 hr. with 0·5 mg./ml.) was found to be identical with the activity of a control sample of the same preparation kept under similar conditions without collagenase. The collagenase used contained an active hyaiuronidase and a little lecithinase; it is to be expected that this latter enzyme would have some effect on any magnesium-activated adenosinetriphosphatase, other than actomyosin, present in the myofibril (Kielley & Meyerhof, 1948, 1950).

Activation of adenosinetriphosphatase by cations. The effect of increasing potassium chloride concentrations on the calcium-activated adenosinetriphosphatase of the myofibril is similar to that obtained with myosin and actomyosin (myosin B). Fig. 5 shows that as the preparations age the general form of the curve is the same, but it is to be noted that the inhibiting effect of potassium chloride concentrations greater than 0·1 M is not so marked (cf. Fig. 2). Myosin B shows a similar effect.

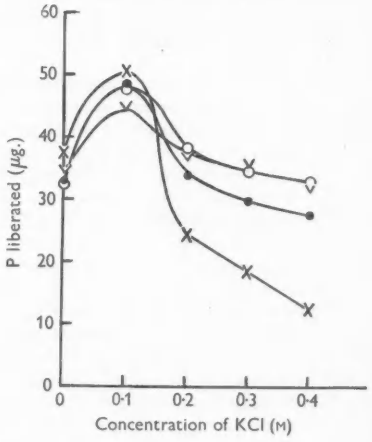

Fig. 5. The effect of KCl on the adenosinetriphosphatase activity of collagenase-prepared myofibrils stored at 0° in borate buffer, pH 7·1. Activity measured in glycine with 0·005 M-$CaCl_2$ at the following times after completion of the preparation: 18 hr., ×; 7 days, ●; 11 days, ○; 18 days, v.

In the absence of cations other than those due to the adenosinetriphosphate and the buffer, maximum activation in glycine, pH 8·6, was reached at

0·01 M-calcium chloride, whereas under the same conditions 0·005 M-magnesium chloride produced an even higher rate of splitting at the maximum of the curve (Fig. 6). Comparable experiments with trypsin-prepared myofibrils indicated maximum calcium chloride activation at 0·002–0·003 M, whereas 0·005 M-magnesium chloride gave a flat maximum at 20–30 % of that obtained with calcium chloride.

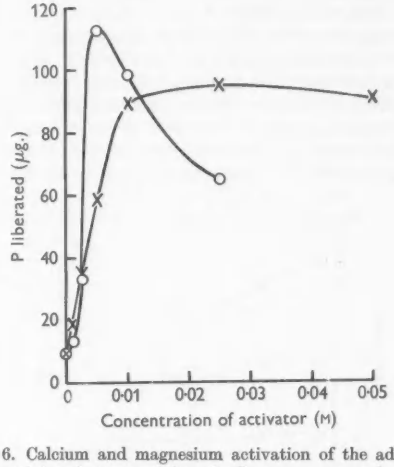

Fig. 6. Calcium and magnesium activation of the adenosinetriphosphatase activity of collagenase-prepared myofibrils in glycine buffer, pH 8·6. $CaCl_2$, ×; $MgCl_2$, ○.

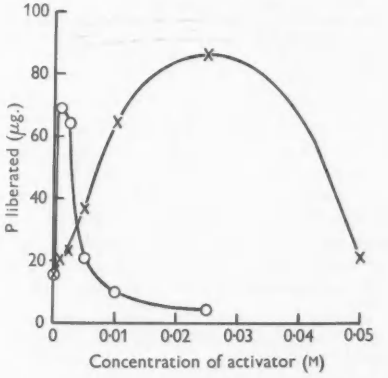

Fig. 7. Calcium and magnesium activation of the adenosinetriphosphatase activity of collagenase-prepared myofibrils in borate buffer, pH 6·9. $CaCl_2$, ×; $MgCl_2$, ○. The myofibril preparation was washed by centrifugation an additional three times.

The activation curves obtained in borate buffer, pH 6·9, are similar to those given with glycine. Fig. 7 shows that at a higher calcium chloride concentration, 0·025 M is required for maximum activation, and the peak with magnesium chloride is sharper and occurs at 0·001–0·002 M. Washing the myofibrils by centrifugation for a further three times did not alter the form of the activation curves at pH 6·9.

The magnesium-activated adenosinetriphosphatase was further investigated to decide whether it was a property of actomyosin or due to another independent enzyme. It is well established that the

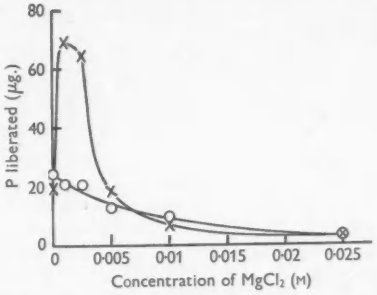

Fig. 8. The adenosinetriphosphatase activity in borate buffer, pH 6·9, with 0·001 M-$MgCl_2$, of actomyosin extracted from collagenase-prepared myofibrils and three times precipitated. In presence of 0·1 M-KCl, ○; no KCl, ×.

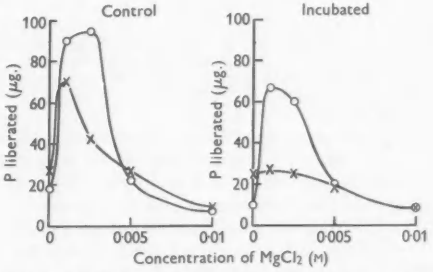

Fig. 9. The effect of incubation for 150 min. at 37° in borate buffer, pH 7·1, on the magnesium-activated adenosinetriphosphatase activity of collagenase-prepared myofibrils. Enzymic activity measured in borate buffer, pH 6·9. 0·1 M-KCl, ×; no KCl, ○.

adenosinetriphosphatase activity of myosin is increased by low concentrations of magnesium in the absence of other cations (cf. Fig. 8), whereas in the presence of potassium chloride magnesium inhibits (Banga & Szent-Györgyi, 1943; Mommaerts & Seraidarian, 1947). On the other hand, the adenosinetriphosphatase activity of myosin, in the absence of actin, is inhibited by magnesium irrespective of the potassium chloride concentration. In contrast to the behaviour of extracted actomyosin,

magnesium invariably produced an increase in the activity of freshly prepared collagenase myofibrils in the presence of 0·1 M-potassium chloride, but this effect tends to disappear on storing, in some cases after a day or two, at 0°. These stored preparations still showed a sharp peak in activity at 0·001 M-magnesium chloride in the absence of potassium chloride. Incubation of the myofibril suspension for 2–3 hr. at 37° had a similar effect to storing, as is shown in Fig. 9, although the latter results were not invariably obtained. Microscopic examination of the incubated myofibrils did not show any obvious morphological change.

Although these results seem to suggest the existence of a separate magnesium-activated adenosinetriphosphatase, it is considered that actomyosin is responsible for all the adenosinetriphosphatase activity, and that the state of organization of the actin and myosin in the myofibril is the important factor. The pH activity curves in Figs. 10 and 11 strongly suggest that one enzyme is responsible for the magnesium-activated adenosinetriphosphatase activity with and without potassium chloride, for with increasing pH, enzymic activities under these two conditions fell off together when the myofibrils were intact. If the myofibrils were first dissolved in M-potassium chloride and additions for the enzyme test arranged so that the final potassium chloride concentration was 0·1 M, 0·001 M-magnesium chloride inhibited; on the other hand, if the intact myofibrils were added directly to the enzyme tube already containing 0·1 M-potassium chloride, the same concentration of magnesium activated (Table 3). Fig. 13 shows that pH has little effect on the magnesium-activated adenosinetriphosphatase activity in the presence of 0·1 M-potassium chloride once the myofibrils have been treated with M-potassium chloride. It is difficult to explain the results on the basis that actomyosin is not as accessible to ions in the intact myofibril as in the dissolved and reprecipitated form, because the substrate readily reaches the former as judged by the rate at which adenosinetriphosphate is split, and both activation and inhibition effects are obtained with potassium chloride acting on the intact myofibril. The calcium-activated adenosinetriphosphatase activity measured in borate at pH 6·9 is not affected by dissolving the myofibrils in M-potassium chloride (Table 3).

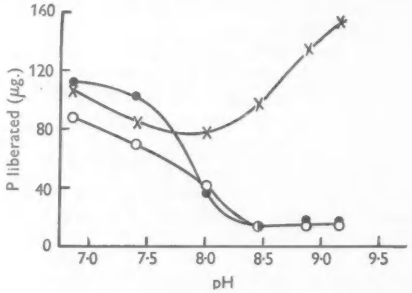

Fig. 10. The effect of pH on the adenosinetriphosphatase activity of intact collagenase-prepared myofibrils in borate buffer. 0·001 M-$MgCl_2$, ●; 0·001 M-$MgCl_2$, and 0·1 M-KCl, ○; 0·005 M-$CaCl_2$ and 0·1 M-KCl, ×.

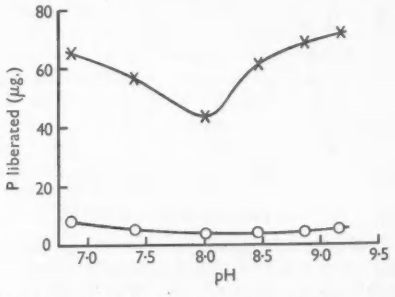

Fig. 11. The effect of pH on the adenosinetriphosphatase activity of collagenase-prepared myofibrils after initial treatment with M-KCl. Enzymic activity measured in borate buffer and 0·1 M-KCl. 0·001 M-$MgCl_2$, ○; 0·005 M-$CaCl_2$, ×.

Adenosinetriphosphatase activity other than that due to the myofibrils. To investigate adenosinetriphosphatase activity in the fraction dispersed in 0·08 M-borate buffer, pH 7·1, after centrifugation, a portion of the collagenase-treated suspension of muscle

Table 3. *Effect of cations on the adenosinetriphosphatase activity of collagenase prepared myofibrils before and after treatment with* M-KCl

(Enzyme activity was measured in borate buffer, pH 6·9.)

Final concentration of activator (M)			ATPase activity (μg. P liberated/ml. myofibrils/20 min.)	
KCl	$CaCl_2$	$MgCl_2$	Intact myofibrils	Myofibrils initially treated with M-KCl
0·1	Nil	Nil	85	88
0·1	Nil	0·001	232	49
0·1	0·005	Nil	308	318

slices was homogenized in the Waring blender, and centrifuged at 1500 *g* until no myofibrils could be detected in the supernatant. This supernatant was removed as completely as possible and the myofibrillar fraction resuspended in a volume of the borate buffer equal to that of the supernatant removed. The adenosinetriphosphatase activities of the supernatant and the resuspended myofibrillar fraction were compared under the following conditions. In borate buffer, pH 6·9, with (*a*) no activator; (*b*) 0·1 M-potassium chloride; (*c*) 0·001 M-magnesium chloride; (*d*) 0·1 M-potassium chloride and 0·001 M-magnesium chloride: and in glycine buffer, pH 8·6, with 0·1 M-potassium chloride and 0·005 M-calcium chloride. From the results it can be concluded that in no case does the myofibrillar fraction contribute less than 97% of the adenosinetriphosphatase-splitting activity of the whole homogenate obtained after collagenase treatment in 0·08 M-borate.

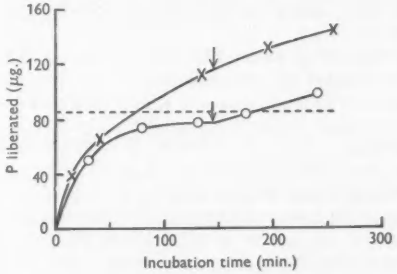

Fig. 12. The splitting of ATP by prolonged incubation with collagenase-prepared myofibrils. Incubation in glycine, pH 8·6, with 0·005 M-$CaCl_2$. The small arrows show the points at which additional amounts of the myofibril suspension were added, and the dotted line indicates 50% of the 10 min. hydrolysable P of the ATP. Normal preparation, ×; normal preparation after three additional washings with 0·08 M-borate, ○.

No magnesium-activated β-glycerophosphate-splitting activity at pH 6·9 or 8·6 could be demonstrated either in the supernatant from homogenized collagenase-treated muscle or by the final purified myofibril preparation.

Myokinase activity. On prolonged incubation at 25°, collagenase-prepared myofibrils split more than 50% of the adenosinetriphosphate acid-labile phosphate. Washing the myofibrils by centrifugation a further three times considerably reduced the myokinase activity, but still a little remained (Fig. 12). Trypsin-prepared myofibrils did not split more than 50% of the acid-labile phosphate after prolonged incubation and addition of fresh myofibril suspension, indicating the absence of myokinase. Presumably myokinase is more sensitive than myosin adenosinetriphosphatase to trypsin.

DISCUSSION

Myosin and adenosinetriphosphatase

The striking fact that myosin can be treated with trypsin until its gross physical properties are considerably changed, yet at the same time retain almost unimpaired the ability to split adenosinetriphosphate, suggests at first sight that adenosinetriphosphatase is not identical with myosin, the main structural component of the myofibril. On the other hand, however, the effects of a variety of sulphydryl reagents (Bailey & Perry, 1947), incubation at 37° and exposure to acid (Perry, 1947), on the adenosinetriphosphatase and actomyosin-forming activities of myosin suggest that these properties are closely related. These latter results are not in complete accord with those of Gilmour & Calaby (1949), who used ninhydrin to oxidize the myosin sulphydryl groups. The only specific criterion of actomyosin formation is the decrease in viscosity obtained when adenosinetriphosphate is added to the complex. Bearing this in mind it is difficult to interpret the experiments of Gilmour & Calaby, for in those cases in which the protein concentration was high enough to give an unambiguous viscosity decrease, addition of adenosinetriphosphate precipitated the mixture of actin and trypsin-treated myosin.

The results of Bailey and Perry can be reconciled with the findings reported here if it is assumed that trypsin breaks down the myosin molecule into smaller fragments which retain the active centres associated with adenosinetriphosphatase activity and actin interaction. These myosin fragments, however, would no longer be asymmetrical enough to produce, on interaction with actin, the gel structure of anastomosed protein filaments. This anastomosed structure is characteristic of normal actomyosin (Astbury, Perry, Reed & Spark, 1947) and can be broken down by adenosinetriphosphate with a pronounced change in viscosity. It should be emphasized that in the experiment with trypsin-treated myosin, a fall in viscosity on addition of adenosinetriphosphate is the only criterion used to indicate actomyosin formation. In the same way as myosin and G-actin are considered to give a complex, G-actomyosin (Szent-Györgyi, 1945), the viscosity of which is not affected by adenosinetriphosphate, trypsin-degraded myosin and F-actin could interact to give a similar complex.

Adenosinetriphosphate and the myofibril

So far as can be ascertained the myofibrils obtained by the collagenase method are little modified from the original intracellular structures. During the preparation they have been washed free of soluble sarcoplasmic protein but still show the characteristic structure assigned to the myofibril by

the work of Hall *et al.* (1946) and Draper & Hodge (1949). The point of action of the collagenase is not clear, but the function of both collagenase and trypsin treatments is to break down the interfibrillar links; possibly the Z bands which extend transversely across the myofibrils binding them together and to the sarcolemma (Draper & Hodge, 1949). It is of interest to note that the Z bands have in the past been suggested to be of a collagenous nature, although Barer (1948) reports that the sarcolemma is not attacked by collagenase. The possibility that a potent factor in breaking down the cell may be the hyaluronidase impurity in the collagenase preparation is being investigated.

The interaction of adenosinetriphosphate and the myofibril is of fundamental interest in the elucidation of the contractile process. Like actomyosin extracted from muscle by salt solutions, the intact myofibril shows the property of splitting phosphate from adenosinetriphosphate, the substance which is unique in causing the myofibril to shorten in a certain ionic medium. How related these properties are is not clear, but it may be significant that shortening is most marked at approximately the magnesium chloride concentration which gives optimum adenosinetriphosphatase activity.

In general it can be concluded that the myofibril splits adenosinetriphosphate at a rate to be expected from the amount of myosin that it contains. The fraction, insoluble in 0·08 M-borate, obtained after collagenase treatment of whole muscle, is responsible for 97 % of the adenosinetriphosphatase activity of the whole. Calculations of the type made by Mommaerts & Seraidarian (1947), on the basis of metal activation experiments with extracted myosin and actomyosin, are liable to be misleading as the work described here and that reported by Steinbach (1947) indicate. It must be concluded, however, if the figures obtained by Lundsgaard (1930) actually do represent phosphate liberated during contraction that there is a serious deficit between the adenosinetriphosphate-splitting power of the isolated myofibril and the amount of phosphate which appears during a short tetanus. Over a lower and more restricted concentration range, magnesium is almost as potent an activator for the myofibrillar adenosinetriphosphatase as is calcium, a fact which must be taken into account in theories of muscle contraction which involve the splitting of ATP.

For reasons given earlier, the magnesium-activated adenosinetriphosphatase of the myofibril is considered not to be a different enzyme, but merely part of the actomyosin complex. Preliminary results indicate that the Kielley-Meyerhof magnesium-activated adenosinetriphosphatase is destroyed during the preparation of the myofibrils, and the possibility that this enzyme is a myofibrillar component is being further investigated. If this enzyme is located in the myofibril, the adenosinetriphosphate-splitting potential of that structure would be considerably increased, but it seems more likely to be a sarcoplasmic component judging from the nature of the preparations and the manner in which they are obtained (Kielley & Meyerhof, 1950).

SUMMARY

1. Trypsin was found to be unsatisfactory for isolating myofibrils from rabbit psoas muscle, and a modified method employing collagenase is described.

2. At 0° low concentrations of trypsin degrade the myofibril and destroy the actomyosin-forming ability of extracted myosin, yet at the same time have little effect on the adenosinetriphosphatase activity.

3. Myofibrils isolated by the collagenase method show detailed fine structure in the electron microscope, and in the presence of adenosinetriphosphate shorten and retain considerable organization of structure.

4. The adenosinetriphosphatase activity of isolated myofibrils is activated by both calcium and magnesium. In the absence of other cations the extent of activation at pH 6·9 and 8·6 by 0·001–0·002 M-magnesium is of the same order as that obtained with optimum calcium concentrations.

5. Unlike extracted actomyosin, intact myofibrils have magnesium-activated adenosinetriphosphatase activity in the presence of 0·1 M-potassium chloride. Destruction of the organization by dissolving the myofibrils in 1·0 M-potassium chloride transforms the magnesium activation effect in 0·1 M-potassium chloride to one of inhibition. This change from activation to inhibition is frequently obtained on storing and also after incubation at 37°.

6. Normal preparations have myokinase activity which can be almost entirely removed by repeated washing at the centrifuge.

7. It is concluded that actomyosin alone is responsible for both the calcium and magnesium activated adenosinetriphosphatase activity of the myofibrils isolated by the collagenase method.

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On Ergothioneine in Blood and Diazo-reacting Substances in Maize

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Ergothioneine, discovered in ergot of rye (Tanret, 1909), was isolated from pig blood by Hunter & Eagles (1925, 1927) and identified as such by Newton, Benedict & Dakin (1926) and by Eagles & Johnson (1927). It was somewhat later prepared from pig blood by Benedict, Newton & Behre (1926). Hunter (1928) described for the substance a delicate and highly specific colour test by which ergothioneine appeared to be present in significant quantity in the blood corpuscles of several animal species. Gulland & Peters (1930) recorded the isolation of ergothioneine from pigeon blood, and Williamson & Meldrum (1932) its isolation from pig blood. Eagles & Vars (1928) brought forward some evidence that the presence of ergothioneine in the blood of the pig was traceable to the diet, and more specifically to some constituent of maize. There has been no confirmation of the work of Eagles & Vars, partly on account of the fact that there was no satisfactory method for the determination of ergothioneine in blood.

The observations of Lawson & Rimington (1947) on an antithyroid activity of ergothioneine revived interest in the substance, and Hunter (1949) has described a new fractionation procedure to obtain blood filtrates suitable for ergothioneine determination by the diazo method.

The amounts of ergothioneine found in human, pig and rat blood are shown in Table 1, and a glance at the mean values shows a wide variation associated apparently with geographical location and particularly apparent in the values shown for pig blood.

The values given for Toronto, are calculated from those published by Hunter (1928). In that paper ergothioneine was expressed as mg./100 ml. corpuscles. In Table 1 of the present paper those values have been halved to give corresponding approximate values for whole blood. It can be seen that pig blood in the Toronto area in 1928 had more than three times the mean ergothioneine content of pig blood in the London area in 1949–50; and at present pig blood in the Edmonton area has about twice the concentration of pig blood in the London area. The relationship between Edmonton (Canada) and London (England) at present is similar with respect to human and rat blood.

There can be little doubt that such variations in ergothioneine blood level are real. They have been

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Table 1. *Ergothioneine in blood (mg./100 ml. whole blood). Author's observations*

Date	Place	Species	No.	Mean	S.D.	Range
1949	Edmonton, Canada	Human	>50	5·3	—	2·5– 9·5
1949	London, England	Human	20	2·9	0·75	1·8– 4·4
1928	Toronto, Canada	Pig	12	13·6	7·5	2·0–26·5
1949	Edmonton, Canada	Pig	>10	7·8	—	6·0– 9·3
1949–50	London, England	Pig	13	4·4	0·74	3·3– 5·3
1949	Edmonton, Canada	Rat	>10	6·1	—	2·9– 8·8
1950	London, England	Rat	15 (males)	1·9*	0·43	1·3– 2·6
1950	London, England	Rat	15 (females)	2·4*	0·53	1·3– 3·1

* This difference is significant; $t = 3{\cdot}12$ when $P < 0{\cdot}01$.

found by the same worker with the same method. The earlier method of Hunter (1928) made no claim to be precise especially for bloods in which the ergothioneine concentration was low, but with the pig bloods commonly handled in Toronto in 1927 the values published are probably substantially accurate, and at any rate represent minimum rather than maximum values. In support of this view it may be noted that Hunter & Eagles (1925) commonly isolated about 0·7 g. of pure ergothioneine from about 10 l. of pig blood, or a yield of 7 mg./100 ml. whole blood; and it is unlikely that such a yield represented more than 50 % of the substance actually present. Hunter, Molnar & Wight (1949) record that in the preparation of ergothioneine from pig blood in Edmonton their best yields were little better than 3 mg./100 ml., and several workers in Britain have failed to prepare ergothioneine from pig blood (personal information). It was also noted by Eagles & Vars (1928) that there appeared to be marked differences in the ergothioneine level of bloods of pigs from different regions of the United States of America.

In view of the wide differences in ergothioneine blood level thus established, and also because of its possible action on the thyroid gland, an attempt to find some explanation for the origin of ergothioneine in blood has here been made. There was strong circumstantial evidence that diets were responsible for the large differences found in the blood level of ergothioneine, and the 'maize theory' of Eagles & Vars (1928) had several points in its favour. Pigs fed maize had a high blood ergothioneine level, and it was suggested by Eagles & Vars (1928) that hydrolysed zein yielded a diazo-giving material that contained a thiolglyoxaline nucleus—possibly in the form of thiolhistidine—as suggested by a positive diazo test (Hunter, 1928, 1930).

Accordingly, it was decided to undertake feeding experiments with pigs and rats, and at the same time to investigate the diazo-giving material extractable from maize meal with methanol. As the present feeding experiments have failed to establish the existence of an ergothioneine precursor in maize, the chemical investigation of maize here undertaken may not appear to be relevant to the main problem of explaining the differences in the level of ergothioneine of bloods. It is useful, however, to know that three distinct substances in maize give diazo tests. As the investigation had to be terminated at the present stage, it has not been possible to identify the substances, but the information obtained may be of some use to future workers in this field.

EXPERIMENTAL

Diazo test. (1) *Diazo reagent* (Koessler & Hanke, 1919). In a 50 ml. volumetric flask immersed in ice water is placed 1·5 ml. of a solution containing 9 g. sulphanilic acid and 90 ml. 37 % HCl/l., followed by 1·5 ml. 5 % $NaNO_2$. Allow to stand 5 min. then add further 6 ml. $NaNO_2$ solution. After further 5 min. make up to volume with ice-cold water and mix. The reagent must be kept cold.

(2) *Alkaline buffer.* 1 g. anhydrous Na_2CO_3 and 10 g. anhydrous sodium acetate in 100 ml. water.

(3) 10 N-*Sodium hydroxide.* If x ml. test solution are used put $2 - x$ ml. water in tube in ice water followed by 1 ml. diazo reagent and 2 ml. alkaline buffer. Add x ml. test solution at once and mix. A clear lemon-yellow colour develops in the presence of ergothioneine. Allow 45 sec. for coupling then remove from ice water and run in 5 ml. 10 N-NaOH and mix. Warm by holding in hand. In 3–4 min. the solution is free of air bubbles and the final purplish-pink colour has fully developed. This colour fades slowly.

Determination of ergothioneine in whole blood. Hunter (1949).

A. *Feeding experiments*

(*a*) *Pigs.* Through the kindness of Prof. H. D. Kay, Director, The National Institute for Research in Dairying, Reading, it has been possible to test the effect of a maize diet on the ergothioneine level of pig blood. Eight female pigs of average weight 45 lb., and from two litters, were divided into two groups of four, one of which was fed diet *M* and the other diet *O* of composition:

	Diet *M*	Diet *O*
Maize meal	77	—
Fine wheat offal	10	45
Barley meal	—	42
Fish meal	10	10
Dried yeast	3	3
Cod liver oil	30 g. once weekly	

Dr R. Braude was in charge of the experiment and sent oxalated blood samples, about 2 ml. from each of the pigs, on the dates specified in Table 2. On both diets the pigs grew at a fairly satisfactory rate, and on 17 April, at the end of 3 months on the diets, the average weight in both groups was 141–144 lb.

The results of the experiment are recorded in Table 2, ergothioneine being determined in the blood by the method of Hunter (1949) from a calibration curve determined for the Pulfrich potometer using filter no. 4.

Comment. For about 3 weeks before the start of the experiment all the pigs had been on a diet containing about 20% maize. It is clear that increasing the maize to 77% in the ration did not increase the ergothioneine in the blood, but did maintain the level better than diet *O*, containing no maize. Although the loss of ergothioneine from the blood on diet *O* is statistically significant, the level of ergothioneine, just over 4 mg./100 ml. whole blood for the pigs on diet *M*, quite obviously does not account for the discrepancies discussed in Table 1, and fails to confirm the suggestion of Eagles & Vars (1928) that dietary maize contains a blood-ergothioneine precursor.

(*b*) *Rats.* Thirty albino rats were taken, fifteen male and fifteen female, that had been raised on a maize-free diet composed of whole wheat 45, Sussex ground oats 40, fishmeal 8, dried skim milk 3, cod liver oil 2, dried yeast 1, and salt 1. Six of the animals, three male and three female were anaesthetized, bloods removed by puncture of the exposed hearts, and the animals killed. The blood ergothioneine values of these control animals are recorded in Table 3.

The remaining twenty-four rats were divided into four cages of six male and six female and fed diets *A* and *B* of composition:

	Diet *A*	Diet *B*
Maize meal	80	—
Methanol-washed maize meal	—	76
Casein	10	10
Olive oil	—	4
Glaxo salt mixture	4	4
Dried yeast	5	5
Cod liver oil	1	1

Maize meal in lots of about 1·5 kg. was washed in a percolator at room temperature with methanol until the extract was almost colourless and no longer gave a positive diazo test. The dried extracted material, free from methanol, is the constituent referred to as methanol-washed maize in rat diet *B*.

On the dates specified in Table 3 one rat was removed from each cage, the blood taken and the animal killed as described for the controls. The ergothioneine values are recorded in Table 3.

Comment. The mean ergothioneine values of the blood of rats fed maize over a period of 15 weeks are

Table 2. *Ergothioneine in blood of pigs fed maize (diet M) and no maize (diet O) (mg. ergothioneine/100 ml. whole blood)*

Ear mark	17 Jan.	30 Jan.	9 Feb.	20 Feb.	27 Mar.	17 Apr.
			Diet *M*			
10†	5·3	5·3	5·3	5·7	5·7	4·8
11†	5·3	4·8	4·8	4·8	4·4	4·0
22‡	4·2	4·0	4·4	4·4	4·4	3·5
14‡	5·3	5·3	5·3	5·3	4·8	4·4
	Mean 5·0*					Mean 4·2*
			Diet *O*			
08†	5·3	4·8	4·0	4·0	3·5	3·3
09†	4·8	4·0	3·5	3·5	2·9	3·5
21‡	4·4	4·0	3·5	3·5	3·3	3·1
23‡	Clotted	4·4	3·5	3·1	3·3	2·9
	Mean 4·8**					Mean 3·2**

* Difference not significant; $t = 1{\cdot}95$, $P > 0{\cdot}05$.
** Difference significant; $t = 6{\cdot}12$, $P < 0{\cdot}01$.
† Litter mates.
‡ Litter mates.

Table 3. *Ergothioneine in blood of rats fed no maize (control), maize (diet A), and methanol-washed maize (diet B) (mg. ergothioneine/100 ml. whole blood)*

Diet	Sex	16 Jan.	26 Jan.	6 Feb.	16 Feb.	15 Mar.	24 Apr.	2 May	
Control	M	1·6, 1·5, 1·3	Mean (6 rats) = 1·6*						
	F	2·2, 1·7, 1·3							
A	M	—	2·4	1·5	2·0	2·4	1·5	1·8	Mean (12 rats) = 2·3*
A	F	—	3·1	2·9	2·6	3·1	2·0	2·2	
B	M	—	2·6	1·8	2·0	2·6	2·0	1·5	Mean (12 rats) = 2·3*
B	F	—	2·9	2·6	2·6	2·9	2·1	2·0	

* This difference is significant; $t = 3{\cdot}3$ or $P < 0{\cdot}01$.
N.B. The last eight values for 24 April and 2 May have mean = 1·9. This is not significantly different from controls; $t = 1{\cdot}84$ or $P > 0{\cdot}05$.

significantly higher than corresponding values on rats fed a maize-free diet, although the mean value of the last eight rats killed was not significantly higher than the controls. There is no evidence that washing the maize meal with methanol removes any ergothioneine precursor. As in the pig experiments, the findings fail to account for the discrepancies noted in Table 1.

B. *Diazo-reacting substances in maize*

It has been observed that, besides ergothioneine, other substances containing the thiolglyoxaline nucleus give a clear yellow colour on coupling with the alkaline diazo reagent, and on the addition of strong base the yellow colour is changed to orange or red (Hunter, 1930). As has been noted above, Eagles and Vars, on the basis of this test, suggested that there was a thiolglyoxaline present in zein. Ashley & Harington (1930) note that they searched for 2-thiolhistidine in zein and I have examined zein, hydrolysed with 20% hydrochloric acid, without finding evidence of the presence of a thiolglyoxaline nucleus.

When a little maize meal powder is mixed with the alkaline diazo reagent used to test for ergothioneine a pinkish colour appears which acquires a more intense red on the addition of concentrated sodium hydroxide solution. This diazo-giving material of maize is extractable with methanol and thus probably not protein in character. Here appeared a possible explanation of the claim of Eagles & Vars that maize contained a dietary precursor of the ergothioneine of blood, which precursor had not been proved to be present in zein. The nature of the diazo-giving material in maize was thought worth investigating while the nutritional tests on pigs and rats, described above, were progressing. It was also for this reason that one group of rats was fed maize that had been freed from the diazo-giving substance by washing with methanol.

Fractionation of methanol extract. The methanol extract from about 1·5 kg. maize meal, vol. 4–5 l., was treated as follows:

The bulk of the methanol was distilled off at atmospheric pressure on a boiling-water bath to reduce the volume to about 500 ml. Distillation was continued *in vacuo* to reduce the aqueous fatty emulsion to about 100 ml. As the maize meal was not dried to begin with there was appreciable water in the extract, but after vacuum distillation it was quite free from methanol. Light petroleum, b.p. 40–60°, was added copiously to the flask and the mixture transferred to a separating funnel. The brown light petroleum layer, containing much fat and giving no diazo test, was discarded. Washing was repeated to remove all light petroleum-soluble material. There was left a light brown solid suspended in an aqueous phase. The latter was separated and collected as a 'water-soluble fraction' which gave a marked diazo test (see below). The particulate material was washed several times with water, and then dissolved in a minimum volume of methanol. A drop of this methanol solution gave a strong red coupling colour with the diazo reagent, and on the addition of conc. NaOH the colour became more intense and of a blue tinge. To the methanol solution about an equal volume of ethanol was added. This produced a copious precipitate with a tendency to gum formation. The methanol-ethanol mixture, which contained all of the diazo-giving material, was evaporated to dryness *in vacuo* and the residue was extracted with absolute ethanol. The ethanol extract was evaporated to dryness and the dry residue extracted with ethyl ether which removed a yellow pigment but no diazo-giving material. The dry light brown friable material was dissolved in about 25 ml. absolute ethanol and hot aqueous $Ba(OH)_2$ was added until there was no further precipitation. The mixture was centrifuged and the material well washed with absolute ethanol.

The precipitate, which contained the substance sought, was now extracted with water which dissolved its Ba salt. CO_2 was passed through the clear yellow solution and the mixture centrifuged and washed with CO_2-saturated water. The white precipitate was now well drained from water and extracted with ethanol. This dissolved the free water-insoluble substance sought and left insoluble $BaCO_3$ and certain Ba salts not freed with CO_2. The ethanol-soluble material was evaporated to dryness *in vacuo* to give an anhydrous residue. Yield about 200 mg. This is referred to as substance 1.

Three separate preparations are described below.

Preparation *C* was obtained by the above method, and preparation *B* by repeating the fractionation with $Ba(OH)_2$, ethanol, water and CO_2. Preparation *A* was obtained by further treatment. The material, about 150 mg. dissolved in about 5 ml. ethanol, was absorbed on a column of Al_2O_3 and well washed with ethanol. No appreciable material was separated in this way. The column was then extracted with 0·5 N-NaOH in ethanol. The clear yellow eluate, giving an intense diazo test, was made just acid with conc. HCl and the NaCl removed. The filtrate, as clear and colourless as water, was brought to dryness *in vacuo*, and the residue extracted several times with water to remove all NaCl. The nearly white precipitate was then dissolved in ethanol and the solution brought to dryness *in vacuo*. The material was again taken up in ethanol and dried to an anhydrous light brown residue that was readily removed from the tube.

After elution with 0·5 N-NaOH in ethanol, considerable brown material was left on the column, so that preparation *A* is purer than *B* or *C*. A small amount of the material was paper chromatographed with a collidine-lutidine mixture. When sprayed with Na_2CO_3 solution a yellow spot was perceptible before application of the diazo reagent, indicating that the substance is intrinsically yellow in alkaline solution. It gave an intense red on application of the diazo reagent. (R_F about 0·95.) Correct conditions for crystallization of the substance have not been determined. It was observed that 11·4 mg. of the substance completely dissolved in 3 ml. 0·02 N-NaOH, but on standing overnight the contents of the test tube became cloudy due to some amorphous material, but also to numerous glistening rectangular plate-like crystals. The pH of such a solution would be near the pK of the substance (see below). All attempts at crystallization by the usual methods have yielded emphatically amorphous precipitates.

Properties of substance 1

M.p.'s: preparation *A*, 120°; *B*, 118–121°; *C*, 120–122° (uncorrected). The substance burns without ash and tests for halide, S and P were negative. (The substance was fused with Na for the S test.) Analyses of the three preparations are as follows:

Preparation *A*:	C, 61·9;	H, 6·5;	N, 6·0%
Preparation *B*:	C, 63·1;	H, 6·6;	N, 5·4%
Preparation *C*:	C, 63·3;	H, 6·6;	N, 5·8%

$C_{13}H_{17}O_4N$ required C, 62·1; H, 6·8; N, 5·6%.

The substance (59·1 mg.) was dissolved in 17 ml. 0·02 N-NaOH, the solution having a pH of 11·3. When titrated with 0·02 N-HCl precipitation began at pH 9·7 and was complete at pH 8·0. The pK is about 9·1.

The substance (45 mg.) was refluxed with 10 ml. 20% HCl for 10 hr. The material never went into solution, but formed black carbonized flecks and all the diazo-giving substance was destroyed.

Solubilities. The substance is soluble in methanol, ethanol and butanol. It is insoluble in water and in dilute and strong acids. It is insoluble in $NaHCO_3$ and Na_2HPO_4, but soluble in Na_2CO_3, Na_3PO_4 and NaOH solutions. It is insoluble in ether, chloroform and light petroleum.

Tests. Molisch, Fehling, biuret and ninhydrin tests were negative. The substance gives a positive xanthoproteic test, a blue colour with uric acid reagents, and a deep-green colour in ethanol with $FeCl_3$. It gives an incipient Millon's test and a burgundy-coloured solution when heated with 50% H_2SO_4.

A few mg. of the substance added to the alkaline Koessler & Hanke (1919) diazo reagent gives an immediate red colour which quickly fades to yellow. However, if 10 N-NaOH solution is quickly added the red colour acquires a bluish tinge and persists for some hours.

With the buffered thiolglyoxaline diazo reagents the behaviour is similar.

Bromine water is rapidly decolorized by the substance. When a slight excess of bromine has been added the solution no longer gives a diazo test.

Dilute $KMnO_4$ solution is rapidly decolorized in presence of H_2SO_4 by the substance. When excess $KMnO_4$ has been added the solution shows a negative diazo test.

Solutions of the substance in NaOH or Na_2CO_3 absorb strongly in the ultraviolet region. Preparations *A*, *B* and *C* were examined in a concentration of 0·8 mg./100 ml. in 2% (w/v) Na_2CO_3 solution. In all cases the maximum optical densities were at 355 mμ., and in a 1 cm. depth, of magnitudes 0·68, 0·63 and 0·61 respectively. After about 12 hr. the peak optical densities were found to have dropped to 75–80% of the values found in the freshly prepared solutions. Whether this drop is due to tautomerism or decomposition was not determined.

When the substance is examined with just enough base to hold it in aqueous solution the absorption maximum is near 290 mμ.

The above evidence, particularly the analyses and absorption spectra of the three separate preparations, would suggest that the substance has been obtained in a state approaching purity. It would appear that the substance is phenolic in character although the possibility of the presence of a carboxyl group is not excluded. The absence of sulphur definitely excludes the presence of a thiolglyoxaline, and a glyoxaline nucleus is also almost certainly excluded on the evidence of the low percentage of nitrogen and on its behaviour towards the diazo reagents.

The behaviour of the substance towards the diazo reagents is atypical with respect to the intensification of colour produced by the addition of strong alkali. This is not usually produced either with phenols or glyoxalines, though a similar behaviour is found with α- and β-naphthol and with resorcinol. The very rapid development of colour with the Koessler & Hanke (1919) reagents is typical of phenols. It should thus be noted that intensification of colour on the addition of a strong base is not in itself indicative of a thiolglyoxaline test; a yellow coupling colour is also required.

The substance would appear to be unusual in composition and properties, and further study is necessary to determine its constitution, but the present evidence suggests that it is not a precursor of ergothioneine.

Water-soluble fraction. The water-soluble fraction obtained as described above gives an immediate brownish-red colour with the diazo reagent which is intensified to a clear red on the addition of 10 N-NaOH. When the water-soluble fraction, suitably diluted and at pH about 4, is treated with 20% (w/v) lead acetate a small yellowish precipitate containing little diazo-giving material is obtained. When the filtrate from this is brought to pH 9·0 by addition of NaOH a copious precipitate is obtained. When this is freed from Pb by H_2SO_4 the solution gives a dull red diazo test, intensified to a bright red with NaOH. The substance giving this test will here be referred to as substance 2. The filtrate from the Pb precipitate, when freed from Pb by H_2SO_4, gives a yellow diazo test with little intensification by NaOH. This will be referred to as substance 3.

Both substances 2 and 3 are precipitable by phosphotungstic acid. When their phosphotungstates are freed quantitatively with $Ba(OH)_2$ and (H_2SO_4) the solution containing substance 2 is neutral and that containing substance 3 is strongly basic.

Some properties of substances 2 and 3, inferred from examination of such solutions, are shown in Table 4.

It would thus appear that both substances are basic in character and that substance 2 is phenolic. The possibility remains that substance 3 is a thiolglyoxaline. Its diazo test is slight evidence for such a possibility, as certain thioglyoxalines show only slight intensification of the yellow coupling colour on addition of strong base.

DISCUSSION

The uniformity of ergothioneine level in the blood of the eight female pigs and of the six control rats used is impressive. The slight downward trend in blood levels of the pigs is also marked by a regularity commonly associated with good analytical methods. In this connexion, however, it should again be emphasized that the low values here recorded are

Table 4. *Some properties of substances* 2 *and* 3

Substance 2	Substance 3
Neutral	Basic
Precipitated with phosphotungstic acid	Precipitated
Precipitated with lead acetate at pH 9·0	Not precipitated
Diazo test, dull-red intensified by NaOH	Yellow, little change with NaOH
Uric acid reagent, blue	No colour
Molisch test, strongly positive	Strongly positive
Decolorizes $KMnO_4$	Decolorizes $KMnO_4$
Boiled with 5 N-HCl, colourless	Rapid blackening with 'humin' formation
Addition of I_2, brown precipitate	Dark-brown precipitate
Dragendorff's reagent, yellow precipitate	Dark-brown precipitate
Spectral absorption band at max. 290 mμ.	No band

higher, by about 1·0 mg., than the real ergothioneine content of the bloods examined as no adjustment has been made for plasma blanks (Hunter, 1949). A significant yellow colour with the diazo reagent is given by plasma, probably due to histidine and tyrosine. On the other hand, it may be stated that no blood has been examined without evidence by the diazo test of the existence in it of some ergothioneine.

In the case of the twenty-four experimental rats it is assumed that the blood level was the same as the six controls. This is a fairly risky assumption as a slight increase in the control values would render insignificant the statistical significance noted in Table 2. But assuming the reality of a statistical significance here, it is relatively uninteresting in the light of the known much greater physiological differences observed in different geographical regions.

The significant sex difference in rats noted in Table 1, although not large, is probably real, and under more favourable conditions might be increased.

It may be noted that the observations on the chemistry of diazo-reacting substances in maize are in accord with the findings in the feeding experiments in so far as they provide no evidence for the existence of a thioglyoxaline nucleus. The possibility remains that American-grown maize differs in composition from that grown in other regions. The source of the maize used in the present experiments could not be traced.

The problem of why bloods should differ so markedly in ergothioneine level thus remains quite obscure. But the subject has scarcely been investigated. A preliminary observation would indicate that when ergothioneine or thiolhistidine is fed to rats, 5 mg./rat for 3 days, the blood ergothioneine 2 days later is not increased.

Pirie (1933) has shown that ergothioneine catalyses the oxidation of cysteine by hydrogen peroxide at pH 2·0, like certain other substances containing the SH-group.

SUMMARY

1. The level of ergothioneine in the blood of the human, pig and rat is much lower in London, England, than in Edmonton, Canada.

2. The thesis of Eagles & Vars (1928) that maize in the diet contains a precursor of ergothioneine of blood has not been confirmed.

3. It has been shown that there are three substances extractable from maize by methanol that give positive diazo tests, none of which is typical of the presence of thiolglyoxaline.

I would express to Prof. C. Rimington my deepest appreciation for inviting me to be a guest in his Department, and my thanks to its staff for assistance in many ways.

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The Conversion of Cyanogen Chloride to Cyanide in the Presence of Blood Proteins and Sulphydryl Compounds

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Aldridge & Lovatt Evans (1946) showed that the physiological effects of cyanogen chloride are similar to those of hydrocyanic acid, and demonstrated that cyanogen chloride is converted to cyanide *in vivo*. The mechanism of this conversion has been studied and the results are given in this paper. Most of this work was carried out during the war years and although incomplete, the results are thought to be of sufficient interest to warrant publication now.

METHODS

Cyanide and thiocyanate were estimated in pure aqueous solution or biological media by conversion to cyanogen bromide (CNBr) followed by reaction with pyridine and benzidine (Aldridge, 1944, 1945). Cyanogen chloride (CNCl) was estimated by the same method with the bromination stage omitted. It is therefore possible by this method to estimate CNCl, cyanide and thiocyanate in the same solution: (*a*) reaction with pyridine-benzidine reagent without bromination estimates CNCl; (*b*) reaction with pyridine-benzidine reagent after bromination estimates CNCl (if present) + HCN + HCNS; and (*c*) aeration for 20 min. to remove CNCl + HCN, followed by reaction with pyridine-benzidine reagent after bromination estimates HCNS. Cyanide and thiocyanate were determined in blood, etc., after precipitation of proteins with trichloroacetic acid (Aldridge, 1944). This method can be used for the determination of CNCl provided the tubes are stoppered during centrifuging. CNCl added to the blood-trichloroacetic acid mixture before centrifuging can be recovered quantitatively. It has been found that there is, at low concentrations (up to 10 μg./ml.), no appreciable loss of cyanide from the phosphate buffer, pH 7·4 at 37°, provided the tubes are stoppered. In view of the volatility of CNCl and its lachrymatory properties, weighing has been avoided in the preparation of solutions. An approximate dilution has been made using ice-cold pipettes and the amount actually added to the reaction mixture determined by analysis of an identical dilution in water. If pure CNCl is not available, a solution may be prepared free from cyanide and chlorine by the method of Klemenc & Wagner (1938). In all solutions when cyanide has been estimated, thiocyanate has also been determined after removal of cyanide by aeration; all but a few estimations with serum were negative. The fact that it has been shown that the substance estimated is completely volatilized by 20 min. aeration and also gives the reaction for CNBr after bromination, serves as a constant check that it is cyanide. When estimating cyanide or thiocyanate in the presence of glutathione, the bromination must be carried out in acid solution (trichloroacetic); if glutathione is brominated at neutral pH a substance is produced which gives the same reaction as CNBr with the pyridine-benzidine reagent.

Pure microcrystalline rat haemoglobin was prepared as follows: 60 ml. of defibrinated rat blood was obtained by decapitation of about twelve rats. The cells were washed twice with three times its volume of saline poured into 120 ml. distilled water, mixed and left in the refrigerator overnight. The crystalline haemoglobin was centrifuged and washed with ice-cold water. To remove stroma the oxyhaemoglobin was converted to the more soluble reduced haemoglobin (Dudley & Lovatt Evans, 1921) by suspending in 200 ml. water and equilibrating with a N_2-CO_2 mixture at about 40°. When all the haemoglobin was in solution, the solution was filtered rapidly through a sintered-glass filter with a wad of cotton-wool above the filter, and the filtrate reoxygenated by bubbling air through the solution; the oxyhaemoglobin soon began to crystallize out in microcrystals. After leaving overnight in the refrigerator, centrifuging down and washing with 50 ml. cold distilled water, it was resuspended in 60 ml. saline phosphate buffer (mix equal volumes 0·165 M-NaCl and 0·165 M-NaH_2PO_4 and bring to pH 7·4 with 0·33 M-NaOH using a glass electrode). This preparation is stable for several weeks in the refrigerator and has a total haemoglobin content of 10–14 vol. O_2%. Pure carboxyhaemoglobin was prepared by a method of Wilson (1945) to whom I am grateful for permission to give the details. Methyl nitrite is generated by dropping 30% (v/v) H_2SO_4 into a mixture of equal volumes of saturated $NaNO_2$ and methanol. Wash the gas free of acid spray by bubbling through dilute $NaHCO_3$. Methyl nitrite (10 ml.) is introduced by a glass syringe into a partly evacuated tonometer containing 20 ml. of haemoglobin suspension. After 5 min. equilibration, evacuate slightly, add a further 10 ml. of methyl nitrite and repeat the equilibration. It is necessary to equilibrate rat haemoglobin suspension longer with methyl nitrite than red cells or haemolysates of red cells of other species owing to the insolubility of rat haemoglobin. The excess methyl nitrite is removed by evacuation and equilibration with air six times. This preparation of methaemoglobin has the advantage that unlike that prepared by the $NaNO_2$ and $K_3Fe(CN)_6$ techniques the blood is not contaminated with excess reagents—methyl nitrite is almost insoluble in water and a few evacuations will remove all excess.

Membranes for ultrafiltration were prepared by the method of Wilson & Holliday (1933).

All reactions studied in this paper were carried out either in the isotonic saline-phosphate buffer given above, or Sørensen's phosphate buffer (M/15) at pH 7·4 and 37°.

All spectrophotometric determinations with haemoglobin derivatives were done using the Unicam S.P. 500 Quartz Spectrophotometer.

RESULTS

Cyanogen chloride is fairly stable at neutral pH, hydrolysing slowly to cyanate and chloride. In addition, about 1 % of the cyanogen chloride is converted to cyanide. After purification of cyanogen chloride, by standing over calcium hypochlorite to remove any cyanide present, followed by distillation over calcium chloride and lime, no reduction of the amount of cyanide produced during hydrolysis is found.

Cyanogen chloride reacts rapidly with both serum and red cells (Aldridge & Lovatt Evans, 1946), but cyanide is produced only from the reaction with red cells. As would be expected in view of this, higher percentage conversions of cyanogen chloride to cyanide are obtained with washed red cells than with whole blood. The presence of cyanide was confirmed by the copper benzidine, Prussian blue and thiocyanate reactions in the trichloroacetic acid filtrate after deproteinization and also in the sodium hydroxide used as an absorbent after volatilization of the cyanide from the untreated blood under vacuum. The formation of cyanmethaemoglobin has also been used to confirm the presence of cyanide.

Haemolysis of red cells produced a slight slowing of the reaction rate while dialysis produced a considerable slowing. Dilution of the original blood during dialysis was corrected by bringing all the solutions to the same haemoglobin content which was checked by the acid haematin method.

Table 1. *Conversion of cyanogen chloride to cyanide by various solutions*

(1 ml. CNCl solution was added to 10 ml. of each solution (final concn. CNCl = 32·3 μg./ml.). All solutions were brought to same haemoglobin content, 11·5 vol. O_2%. All solutions except whole blood were in M/15 phosphate buffer pH 7·4 and at 37°.)

	HCN produced (μg.)		
	10 min.	3 hr.	5·75 hr.
Whole blood	26	26	26
Red-cell suspension	36	35	36
Haemolysed red cell	28	31	36
Dialysed red cell	19	25	27

Reduction of the haemoglobin of whole blood and washed red cells by repeated evacuation and equilibration with nitrogen increases the amount of cyanogen chloride converted to cyanide by 15–20 %.

Cyanogen chloride reacts rapidly with rat haemoglobin, no more than 1 % of the original cyanogen chloride added being left after 5 sec. from a mixture of cyanogen chloride (17·3 μg./ml.) and of haemoglobin (5–7 vol. O_2 %). When higher concentrations of cyanogen chloride are used, i.e. 50–100 μg./ml., there is an extremely rapid removal of cyanogen chloride in the first 5 sec. followed by a slower reaction. This is probably due to only a small amount of the haemoglobin being in solution. No cyanide is liberated during this initial reaction with haemoglobin. However, if haemolysed red cells (with or without cell stroma) are added to this reaction mixture there is an immediate liberation of cyanide. Addition of plasma or serum produces no cyanide and intact red cells liberate cyanide very slowly from a reaction mixture of cyanogen chloride and haemoglobin. In these experiments cyanide has therefore been produced by a two-stage reaction, the first an extremely rapid one of cyanogen chloride with haemoglobin followed by a slower reaction with some substance present in the red cell and not in the plasma. Oxy-, carboxy- and reduced haemoglobin all react with cyanogen chloride to give a product which releases cyanide on reaction with haemolysed red cells. On the other hand, methaemoglobin reacts with cyanogen chloride, but no cyanide is liberated from the product on reaction with haemolysed red cells. Any cyanide formed would react with the methaemoglobin to form cyanmethaemoglobin; however, cyanide may be estimated in methaemoglobin solutions after the usual trichloroacetic acid deproteinization. Addition of haemolysed red cells to the reaction product of serum and cyanogen chloride does not produce any cyanide. The formation of a compound with cyanogen chloride from which cyanide may be produced is therefore peculiar to haemoglobin among the proteins present in blood.

The addition of the solution obtained by centrifuging red cells after treatment with cyanogen chloride, to methaemoglobin, shows the immediate formation of cyanmethaemoglobin (disappearance of the band at 625 mμ. and formation of a band at 539 mμ.) thus confirming the presence of cyanide. The addition of cyanogen chloride to oxyhaemoglobin and methaemoglobin produces no immediate change of visible spectrum. However, cyanogen chloride appears to react slowly with methaemoglobin to produce cyanmethaemoglobin. After 1 hr. the amount of cyanmethaemoglobin formed in the presence of 35 μg./ml. cyanogen chloride was roughly equivalent to that produced in 5 min. from 0·8 μg./ml. cyanide. It therefore seems probable that the reaction of cyanogen chloride with haemoglobin followed by the liberation of cyanide from this compound does not involve reaction with the haem portion of the haemoglobin molecule. Throughout this paper, therefore, the product of the interaction of cyanogen chloride with haemoglobin has been referred to as CNCl/globin. This has been used instead of CNCl/Hb to avoid confusion with other derivatives of haemoglobin, e.g. cyanmethaemoglobin Met Hb/CN.

An attempt was made to find the essential component for the second stage in the reaction. Sodium fluoride (10^{-2}M) and sodium cyanide (3×10^{-4}M was the maximum we could use for practical reasons) added to haemolysed red cells did not reduce the liberation of cyanide from the CNCl/globin. Heat denaturation of haemolysed red cells yielded an active protein-free filtrate, but the rate of liberation of cyanide was appreciably lowered. An enzyme cannot, therefore, be involved in the reaction. Dialysed red cells gave a definite but slower liberation and a smaller total liberation of cyanide. Ultrafiltrate of haemolysed red cells was active, though if a trace of copper was added and the solution shaken with air, no cyanide was produced. Table 2 shows that in these experiments there was a correlation between the presence of free SH groups and the liberation of cyanide from the CNCl/globin.

Reaction of cyanogen chloride with glutathione

The number of molecules of cyanogen chloride reacting with one molecule of glutathione (Table 3) has been calculated from the titration of —SH groups before and after reaction with cyanogen chloride, by the method of Lucas & King (1932). One molecule of cyanogen chloride reacts with one molecule of glutathione in the initial fast reaction. When the —SH compound is in excess the initial reaction is followed by a liberation of cyanide (Table 4). If, however, all the free —SH groups are removed by reaction with excess cyanogen chloride, the excess cyanogen chloride being removed by aeration, no cyanide is produced even after 4 hr. incubation. Addition of further glutathione causes an immediate liberation of cyanide (Table 5). This indicates that —SH groups are essential for the liberation of cyanide

Table 2. *Correlation between presence of* —SH *groups and liberation of cyanide from* CNCl/*globin compound*

(CNCl/globin compound prepared by adding 1 ml. CNCl solution (284 μg.) to 5 ml. haemoglobin solution (approx. 10 vol. O_2%). After 10 min. 2 ml. of this mixture were added to 2 ml. of the various solutions. Nitroprusside test was carried out on tungstic acid filtrates.)

Reaction mixture	Percentage conversion CNCl→HCN: 10 min.	4 hr.	Nitroprusside test
CNCl/globin + haemolysed red cells } Hb concn. 5·7 vol. O_2%	33	67	+ + + +
CNCl/globin + dialysed red cells } Hb concn. 5·7 vol. O_2%	—	10	+
CNCl/globin + ultrafiltrate of red cells	8	17	+ +
CNCl/globin + ultrafiltrate of red cells + Cu and shaken with air	—	3	—

The second reaction may therefore be mediated through some substance containing SH groups, which is dialysable and is normally present in red cells, but not in plasma. Glutathione added to the CNCl/globin produces cyanide while cysteine, cystine or ergothioneine do not. It would therefore appear that the essential reactions in the production of cyanide from cyanogen chloride and blood are an initial reaction with haemoglobin followed by liberation of cyanide by reaction with glutathione. However, cyanogen chloride reacts directly with glutathione. The rate of the reaction is of the same order as with haemoglobin, the concentration of cyanogen chloride added being reduced to 1% in 5 sec. From the product of this reaction cyanide is liberated at a slow rate, as it is from the CNCl/globin. Since the rate of reaction of cyanogen chloride with haemoglobin and with glutathione is of the same order, it is possible that both reactions contribute to the conversion of cyanogen chloride to cyanide *in vivo*.

The reaction between glutathione and cyanogen chloride has been examined more fully since it also appeared to be a two-stage process similar to that with haemoglobin, as well as the first stage of the reaction. From titration of —SH groups after the initial reaction and after 4 hr. incubation at 37°, it has been calculated that one molecule of glutathione reacts with the initial reaction product to give one molecule of cyanide (Table 3). It should be noted that in this experiment the —SH groups of the glutathione were protected from atmospheric oxidation during the 4 hr. incubation by the presence of cyanide which forms complexes with traces of catalytic metals.

In view of the above reactions other —SH compounds were examined. Cysteine, the parent —SH amino-acid in glutathione, and *N*-acetylcysteine prepared by the method of Pirie (1931), a substance with an acetyl group in the same position as the glutamyl group on the amino group in glutathione, have been examined. The molecular proportions of the initial reaction with cyanogen chloride were determined by allowing an excess of cyanogen chloride to react with a known amount (5 mg.) of —SH compound, and the amount of cyanogen chloride remaining after the reaction determined. This was done in preference to the determination of —SH groups, in view of the difficulty of obtaining consistent results from the iodine titration of cysteine

Table 3. *Molecules of cyanogen chloride reacting with one molecule glutathione, cysteine or* N-*acetylcysteine*

(1) Reduced glutathione (GSH, 10 mg.) in 5 ml. M/15 phosphate buffer pH 7·4 added to 5 ml. buffer containing an estimated amount of cyanogen chloride. The mixture was incubated at 37° for 10 min. and excess —SH groups were then determined by titration with iodine.

(2) Cysteine (CySH) or N-acetylcysteine (5 mg.) in 5 ml. M/15 phosphate buffer pH 7·4 were added to excess cyanogen chloride in 5 ml. buffer and the mixture incubated at 37° for 10 min. The amount of CNCl remaining was then determined.

The liberation of HCN (slow reaction) was examined by determining further loss of —SH groups by iodine titration and the production of HCN after 4 hr.

(The —SH groups are protected from oxidation during incubation by the presence of HCN.)

	Immediate reaction 10 min., 37°			Slow reaction 4 hr., 37°		
	—SH compound reacting (mg.)	CNCl reacting (mg.)	Molecules CNCl/molecule SH compound	—SH compound reacting (mg.)	HCN produced (mg.)	Molecules SH compound/ molecule HCN
Glutathione	5·52	1·14	1·03	—	—	—
	5·82	1·17	1·0	—	—	—
	4·51	0·952	1·05	2·45	0·226	0·96
	5·45	1·15	1·05	2·68	0·227	1·04
Cysteine	5·0	2·04	1·04	—	—	—
	5·0	1·90	0·98	—	—	—
N-Acetylcysteine	5·0	1·92	1·02	—	—	—

Table 4. *Liberation of* HCN *from product of the reaction of cyanogen chloride with glutathione, cysteine and* N-*acetylcysteine*

(Excess —SH compound in 5 ml. M/15 phosphate buffer pH 7·4 added to 5 ml. CNCl solution (amount estimated given in brackets). HCN was determined at various times.)

Glutathione: 5 ml. GSH solution (5 mg.) +5 ml. CNCl solution (291 μg.)		Cysteine: 5 ml. CySH solution (5 mg.) +5 ml. CNCl solution (810 μg.)		*N*-Acetylcysteine: 5 ml. *N*-acetylcysteine solution (6 mg.) +5 ml. CNCl solution (312 μg.)	
Time (min.)	Conversion CNCl→HCN (%)	Time (min.)	Conversion CNCl→HCN (%)	Time (min.)	Conversion CNCl→HCN (%)
10	12·0	10	4·8	10	4·7
20	20·6	20	4·5	20	6·7
60	43·8	60	4·2	60	9·1
120	64·2	90	4·0	180	17·8
240	62·7	110	To 5 ml. add 5 ml. GSH solution (5 mg.)	200	To 5 ml. add 5 ml. GSH solution (5 mg.)
		120	4·9	210	23·1
		170	4·1	220	25·0
				260	35·5
				320	44·2

Table 5. *Reaction of glutathione and* N-*acetylcysteine with cyanogen chloride*

(—SH compound in 5 ml. phosphate buffer pH 7·4 added to excess CNCl in 5 ml. buffer. Absence of —SH groups was shown by iodine titration after 10 min. incubation at 37°. Excess CNCl was then removed by aeration for 15 min. (absence of CNCl shown by pyridine-benzidine reagent) and the mixture then incubated at 37°. Samples were removed for HCN estimation at various times. Zero time is the time of the original addition of CNCl.)

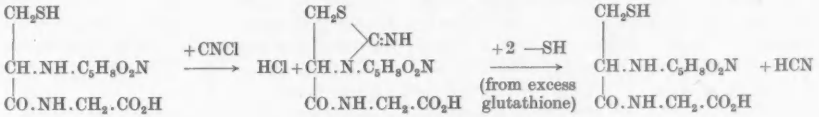
Glutathione: 5 ml. GSH solution (10 mg.) +5 ml. CNCl solution (2·04 mg.)		*N*-Acetylcysteine: 50 ml. *N*-acetylcysteine solution (5 mg.) +5 ml. CNCl solution (2·17 mg.)	
Time (min.)	Conversion CNCl→HCN (%)	Time (min.)	Conversion CNCl→HCN (%)
40	0·8	60	1·3
60	0·8	210	1·3
180	0·9	230	To 5 ml. add 5 ml. GSH (10 mg.)
200	To 5 ml. add 5 ml. GSH (10 mg.)	240	5·5
220	17·1	260	16·5
280	47·4	290	22·5
340	52·8	350	36·5

and *N*-acetylcysteine (Lucas & King, 1932). One molecule of cyanogen chloride reacts with one molecule of both cysteine and *N*-acetylcysteine (Table 3). However, no cyanide is liberated from the reaction product with cysteine even if glutathione is added (Table 4). *N*-Acetylcysteine, on the other hand, liberates cyanide on further incubation. This release of cyanide is rather slow, but is accelerated by the addition of glutathione (Table 4). Like the reaction with glutathione, if all —SH groups are initially removed by reaction with excess cyanogen chloride, no cyanide is liberated until a further supply of —SH groups (glutathione in this case) is provided (Table 5).

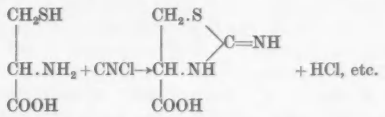
DISCUSSION

In a previous paper (Aldridge & Lovatt Evans, 1946) it was shown that except for the local irritation of the respiratory passages, the physiological response to cyanogen chloride can be explained by its conversion to cyanide in the body. Immediately after the inhalation of cyanogen chloride, cyanide could be detected and determined in the circulating blood, and when cyanogen chloride was added to whole blood *in vitro* there was an immediate appearance of cyanide. The site of this reaction is the red cell, and evidence has been presented that the reaction is a two-stage one, the initial one with haemoglobin giving a product from which cyanide is liberated by glutathione. Subsequently, however, glutathione was shown to react directly with cyanogen chloride to produce cyanide. This reaction also takes place in two stages, the initial reaction being rapid giving a product which liberates cyanide relatively slowly. With intact red cells the production of cyanide from cyanogen chloride is rapid (Aldridge & Lovatt Evans, 1946). This is probably not only due to the high concentrations of haemoglobin and glutathione, but also to the physical conditions therein. We believe, therefore, that both of these mechanisms contribute *in vivo* to the production of cyanide from cyanogen chloride.

From a study of the characteristics of the reaction of glutathione with cyanogen chloride, a reaction similar to that postulated for the reaction of cyanogen chloride with 1:2-dithiols (Aldridge, 1948) can now be suggested:



The evidence for such a reaction is as follows: (*a*) The initial reaction results in the removal of one —SH group per molecule of cyanogen chloride. (*b*) Excess cyanogen chloride, removing all free —SH groups, prevents cyanide liberation and blocks the reaction at stage 2. Addition of glutathione to provide the necessary two atoms of hydrogen results in the immediate liberation of cyanide. (*c*) After the initial reaction one —SH group is removed during the liberation of one molecule of cyanide. According to the above reaction two molecules of glutathione supply two atoms of hydrogen, while one molecule of glutathione is regenerated during the reaction. Since glutathione reacts in this way, cysteine should also react in a similar manner.



However, although it has been shown that one molecule of cyanogen chloride reacts with one molecule of cysteine, no cyanide is produced either in the presence of excess cysteine or when glutathione is added. This difference between the reactions of glutathione and cysteine could be accounted for by suggesting that the ring structure formed from cysteine is more stable than that from glutathione which has the amino group substituted with a glutamyl group. In order to check this point *N*-acetylcysteine has been prepared; this compound should react with cyanogen chloride to produce a structure similar to that with glutathione; this should release cyanide in the presence of excess *N*-acetylcysteine or glutathione. It has been found that this is the case. One molecule of cyanogen chloride reacts with one molecule of *N*-acetylcysteine followed by the liberation of cyanide in the presence of the free —SH groups of *N*-acetylcysteine or of glutathione. If all free —SH groups are removed by reaction with excess cyanogen chloride, no cyanide is liberated until glutathione is added. It therefore seems that vicinal —SH and ⟩NH groups are necessary for the production of cyanide from cyanogen chloride by these reactions.

It has been shown that cyanogen chloride reacts with glutathione, *N*-acetylcysteine, haemoglobin and carboxyhaemoglobin to produce compounds which liberate cyanide on treatment with more glutathione. It is possible that the reaction with haemoglobin is analogous to the initial reactions with glutathione and *N*-acetylcysteine, cyanogen chloride reacting with vicinal —SH and ⟩NH groups

in the globin. Mirsky & Anson (1936) have shown that the available —SH groups in native horse haemoglobin are oxidized by treatment with potassium ferricyanide (i.e. in the conversion of haemoglobin to methaemoglobin). It is probable that treatment with methyl nitrite will also effect a similar oxidation of —SH groups, thus giving an explanation why cyanogen chloride does not react with methaemoglobin to give a product which liberates cyanide on reaction with glutathione. If this hypothesis is correct further work might enable the reaction with cyanogen chloride to produce cyanide to be used as a test for vicinal —SH and >NH groups in enzymes and proteins in general.

It is interesting to note that, in common with the findings of Dixon (1947) that lachrymators react with —SH groups, cyanogen chloride reacts with —SH groups and is also a lachrymator.

SUMMARY

1. Cyanogen chloride reacts rapidly with haemoglobin and with glutathione to produce compounds which on treatment with more glutathione liberate cyanide.

2. It is believed that both reactions contribute to the conversion of cyanogen chloride to cyanide *in vivo*.

3. From a comparison of the reaction of cysteine and the reactions of glutathione and *N*-acetylcysteine with cyanogen chloride a mechanism for cyanide production has been suggested.

4. A possible mechanism of the reaction of haemoglobin with cyanogen chloride is discussed.

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The Effect of the Ingestion of a Hypolipotropic Diet on the Level of Pseudo-Cholinesterase in the Plasma of Male Rats

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In 1943, Faber advanced the hypothesis that the liver serves as the principal locus for the synthesis of plasma cholinesterase (ChE) on the basis of his finding that disturbances of hepatic origin in humans were accompanied by a depression in the activity of plasma towards acetylcholine. This hypothesis has been substantiated by the experimental results of many workers. Brauer & Root (1946) and Ellis, Sanders & Bodansky (1947) found that the plasma of rats receiving carbon tetrachloride, a hepatotoxic agent, showed a decreased ability to hydrolyse acetylcholine. Moreover, the results of Wescoe, Hunt, Riker & Lett (1947), as well as those of Grob, Lilienthal, Harvey & Jones (1947), demonstrated that in human subjects with hepatic insufficiency, the rate of regeneration of plasma cholinesterase following the administration of di*iso*propyl fluorophosphonate was much lower than in subjects with normal hepatic function. Since the cholinesterase activity of human plasma is due predominantly to the activity of pseudo-cholinesterase (Mendel, Mundell & Rudney, 1943), it would therefore seem that the synthesis of pseudo-cholinesterase takes place mainly in the liver and is impaired by hepatic insufficiency.

Accumulation of fat in the liver occurs when the diet is inadequate with respect to its content of lipotropic factors (choline, Best, Hershey & Huntsman, 1932; betaine, Best & Huntsman, 1932; methionine, Tucker & Eckstein, 1937). In animals

with an average liver fat content of 25–30% (resulting from a hypolipotropic diet), impairment in bromsulphalein elimination and an elevated plasma phosphatase level is to be found in a high proportion of the cases (MacLean, Ridout & Best, 1937; McKibbin, Thayer & Stare, 1944).

Accordingly, it was expected that in animals showing an accumulation of fat in the liver pseudo-cholinesterase activity of the plasma would be lowered. The experiments to be reported here reveal that in male rats this does not occur; there is on the contrary a highly significant elevation in the ChE activity of the plasma brought about by a rise in pseudo-cholinesterase activity, but not in true cholinesterase activity.

METHODS AND MATERIALS

Rats of the Wistar strain were used throughout. They were housed in individual cages provided with a false bottom of coarse wire screen. Three days following their setting out, they were divided into the required number of groups balanced with respect to weight.

The diet was weighed out daily and the following morning the amount left over and the scatter were weighed. From these data the individual daily food consumption was calculated. The rats were 'group-pair fed' on a twice weekly basis.

The composition of the basal diet, inadequate with respect to its lipotropic content, is given in Table 1.

Table 1. *Basal hypolipotropic diet*

Component	%
Extracted peanut meal*	30
Casein	6
Sucrose	39
Beef dripping	15
Salt mixture†	4
Corn oil	5
Vitamin powder‡	1
α-Tocopherol acetate	0·010
C.L.O. concentrate§	0·015

* The peanut meal was exhaustively extracted with dilute ethanol and finally dried with absolute ethanol. The choline content was thereby reduced to between 0·2 and 0·5 mg. choline/g., as determined by microbiological assay.

† See Beveridge & Lucas (1945).

‡ The vitamin powder consisted of aneurin hydrochloride, 500 mg.; riboflavin, 250 mg.; pyridoxine, 200 mg.; calcium pantothenate, 1000 mg.; nicotinic acid, 1000 mg.; folic acid, 50 mg.; *p*-amino benzoic acid, 10 g.; 4-methyl 1:4-naphthoquinone, 100 mg.; and inositol, 50 mg. This was made up to 1000 g. with finely powdered sucrose. Rats eating 10 g. of food/day thus received 50 μg. of aneurin and proportional amounts of the other vitamins daily.

§ The cod-liver oil concentrate (Ayerst, McKenna & Harrison, Ltd., Montreal) contained 200,000 i.u. vitamin A and 50,000 i.u. vitamin D/g.

The animals were maintained on the diet for 4 weeks, usually, and then sacrificed by exsanguination through the carotid artery under light ether anaesthesia. Blood samples were collected in the presence of a small amount of sodium oxalate, centrifuged immediately and the plasma drawn off for estimating enzymic activity. The livers were immediately removed, weighed and homogenized in acetone for subsequent estimation of lipids (see Best, Lucas, Patterson & Ridout, 1946). When pseudo-cholinesterase estimations were carried out on the livers, the original homogenate was prepared with water, and samples were taken both for enzymic determinations as well as lipid estimations.

Cholinesterase activity was measured manometrically in 0·025 M-$NaHCO_3$ as medium saturated with 5% CO_2 in N_2 (pH 7·4). Since rat plasma contains both true cholinesterase and pseudo-cholinesterase (Mundell, 1944), the activity of these enzymes was measured separately, using acetyl-β-methylcholine (Mch) (0·03 M) and benzoylcholine (Bch) (0·006 M), respectively, as substrates, according to the method outlined by Mendel *et al.* (1943). The presence of benzoylcholinesterase (Sawyer, 1945) was excluded by the fact that the activity towards benzoylcholine was susceptible to inhibition by $2·5 \times 10^{-6}$ M-eserine (see Hawkins & Gunter, 1945).

The activity of other esterases (ali-esterases) which, like pseudo-cholinesterase, can contribute to the hydrolysis of non-choline esters (Easson & Stedman, 1937), was estimated by measuring the rate of hydrolysis of tributyrin (0·008 M) in the presence of 5×10^{-6} M-eserine, a concentration which completely suppresses the activity of pseudo-cholinesterase while leaving that of other esterases towards this substrate unimpaired.

EXPERIMENTAL

(*a*) *The effect of the ingestion of a hypolipotropic diet on the level of pseudo-cholinesterase in the plasma of adult male rats*

Forty male rats (200–250 g. body weight) were divided into two comparable groups. Group I received the basal hypolipotropic diet, group II the same diet with added choline chloride (0·40%). The experiment was allowed to proceed for 4 weeks, after which time the animals were sacrificed.

Table 2 shows the mean level of activity of pseudo-cholinesterase and true cholinesterase in the plasma of these two groups of rats, together with the average total lipid content of the livers of these animals. It will be seen that the activity of true cholinesterase and of common esterase shows no significant variation under these conditions, whereas that of pseudo-cholinesterase is greatly increased when an inadequate supply of lipotropic factors is provided in the diet. This increase is highly significant, a P value of $5·5 \times 10^{-10}$ being obtained upon analysis of the data.

(*b*) *Plasma and liver pseudo-cholinesterase levels of adult male rats fed a hypolipotropic diet for various periods of time*

The preceding experiment was repeated using forty-eight animals (200–250 g.) on the basal diet and twenty-four of comparable weight on the supplemented diet. At the end of the second, third and fourth weeks, respectively, sixteen of the former and eight of the latter were killed and blood samples taken for analysis. At the same time estimations

Table 2. *The effect of the ingestion of a hypolipotropic diet and of the same diet supplemented with choline on the activity of the cholinesterases in the plasma of adult male rats*

Group	Diet	Average liver wt. (%)	Average total liver lipids (%, wet wt.)	Plasma cholinesterase activity*		Plasma common esterase activity‡
				Pseudo-ChE	True ChE	
I	Basal	19·7	30·1	79·8±4·5	58·9±5·1	1850±140
II	Basal + choline chloride (0·4%)	12·2	6·6	36·2±2·7† $t=6·7$ $P=5·5\times10^{-10}$	59·2±3·0 $t=0·04$ $P=>1$ (not significant)	1670±114 $t=1·02$ $0·3<P<0·4$ (not significant)

* Activity is expressed as μl. CO_2 evolved in 20 min./ml. plasma.
† The values found in the case of the animals maintained on the choline-supplemented diet are in the same general range as those observed in normal animals (see Mendel, Hawkins & Nishikawara, 1948).
‡ Activity expressed as μl. CO_2 evolved from tributyrin (0·008 M) in 20 min./ml. plasma. Eserine (5×10^{-6} M) was added to suppress the activity of pseudo-cholinesterase.

Table 3. *The effect of the ingestion of a hypolipotropic diet for various periods of time on the level of liver and plasma pseudo-cholinesterase activity of adult male rats*

Group	Diet	Duration of ingestion (weeks)	Average total liver lipids (%, wet wt.)	Liver pseudo-ChE activity (Q_{Bch})*			Plasma pseudo-ChE activity†		
				Mean±S.E.	t	P	Mean±S.E.	t	P
I	Basal	2	26·1	2·98±0·20	2·7	0·007	49·4±3·3	4·6	<0·001
II	Basal + choline chloride (0·4%)	2	7·3	2·13±0·19			25·8±1·9		
III	Basal	3	28·13	3·08±0·13	3·1	<0·001	66·2±7·5	3·4	<0·001
IV	Basal + choline chloride (0·4%)	3	6·35	2·31±0·25			27·7±1·6		
V	Basal	4	27·09	3·85±0·23	4·9	<0·001	71·7±6·3	4·3	<0·001
VI	Basal + choline chloride (0·4%)	4	6·50	2·16±0·15			29·5±3·2		

* Q_{Bch} indicates the μl. CO_2 evolved/mg. fat-free dry residue of the liver homogenate/hr.
† Activity expressed as μl. CO_2 evolved/ml. plasma/20 min.

were made of the pseudo-cholinesterase activities of the livers. For this purpose the livers were rendered as free of blood as possible by perfusion with saline through the left ventricle under pressure. They were immediately removed and homogenized with water, with samples of the homogenates being taken for lipid estimations, determinations of enzymic activity and dry-weight estimations. The pseudo-cholinesterase activity of the homogenate was expressed in terms of μl. CO_2 evolved/mg. of the dry fat-free residue/hr. (Q_{Bch}); controls for non-enzymic hydrolysis were run simultaneously.

The results of this experiment are outlined in Table 3. It will be seen from these results that the activity of the pseudo-cholinesterase in the plasma is elevated after 2 weeks' ingestion of a hypolipotropic diet and the level of enzymic activity continues to rise as the period of ingestion of the diet is prolonged. Moreover, the pseudo-cholinesterase activity of the liver (Q_{Bch}) in the animals fed this diet is also above normal, and the level of this enzyme also continues to rise as the period of ingestion of the experimental diet is prolonged.

(*c*) *The effect of the ingestion of a hypolipotropic diet on the plasma pseudo-cholinesterase activity of weanling male rats*

It has been shown (Griffith & Wade, 1939) that weanling rats maintained on a hypolipotropic diet show an elevated liver fat content within 48 hr. and the amount of liver fat increases for the first 4–6 days, during which time there is no evidence of interference with appetite or growth. It was therefore decided to investigate the plasma pseudo-cholinesterase levels of weanling male rats maintained on a hypolipotropic diet to see if the enzymic activity of the plasma of these animals, which showed liver fat accumulation as did the adult rats in the previous experiments, also showed a corresponding elevation.

Twenty male rats (32–40 g. body weight) were divided into two comparable groups. One half received the hypolipotropic ration, the other the same diet supplemented with choline chloride (0·35%). They were group-pair fed on a daily basis for 6 days, and then killed in the usual manner. Gross examination revealed very fatty livers in the

Table 4. *The effect of the ingestion of a hypolipotropic diet in the level of pseudo-cholinesterase in the plasma of weanling male rats*

Group	Diet	Plasma pseudo-cholinesterase activity* Mean ± S.E.	t	P
I	Basal	38·4 ± 3·7	3·1	$2·3 \times 10^{-3}$
II	Basal + choline chloride	25·5 ± 2·7		

* Activity expressed as μl. CO_2 evolved/ml. plasma/20 min.

Table 5. *The ability of various substitutes to prevent the rise in plasma pseudo-cholinesterase observed in male rats maintained on a hypolipotropic diet*

Group	Diet	Average total liver lipids (%, wet wt.)	Plasma pseudo-cholinesterase activity* Mean ± S.E.	t	P
N-0-1	Basal	31·0	81·3 ± 8·3	7·0	<0·001
N-1	Basal + methionine (0·5%)	7·3	28·6 ± 1·7		
N-0-2	Basal	29·2	96·6 ± 6·9	7·7	<0·001
N-2	Basal + betaine HCl (1%)	6·9	38·6 ± 3·0		
N-0-3	Basal	30·5	90·9 ± 8·9	2·3	0·037
N-3	Basal + triethyl homologue of choline (0·5%)	6·3	56·1 ± 7·4		
N-0-4	Basal	31·8	77·9 ± 8·5	1·9	$7·5 \times 10^{-2}$ (not significant)
N-4	Basal + cystine (0·4%)	26·4	102·0 ± 8·5		
N-0-5	Basal	32·8	78·3 ± 7·8	0·67	Greater than 0·5 (not significant)
N-5	Basal + fivefold increase in vitamin content of basal diet	27·5	84·2 ± 4·1		

* Activity expressed as μl. CO_2 evolved/ml. plasma/20 min.

animals without a choline supplement. Table 4 shows that the plasma pseudo-cholinesterase activity of the animals maintained on this basal diet exceeded that found in the animals ingesting the diet containing the choline supplement and the rise was statistically significant.

(d) The effect of various supplements on the elevated plasma pseudo-cholinesterase of adult male rats maintained on a hypolipotropic diet

To determine whether choline is specific in preventing the rise in plasma pseudo-cholinesterase observed in the previous experiments, the effect of various other supplements to the basal diet was investigated. The following five supplements were substituted for a corresponding amount of sucrose in the basal ration: methionine (9·5%), betaine (1%), the triethyl homologue of choline (0·5%), cystine (0·4%) and a fivefold increase in the vitamin content of the basal ration. It was hoped that the levels of methionine and betaine chosen would possess a lipotropic effect comparable to that of the choline used previously. The inclusion of cystine in the scheme was to determine whether the possible efficacy of methionine in reducing the level of the enzyme to normal was due to its action as a sulphur-containing amino-acid or to its formation of choline by transmethylation (du Vigneaud, Chandler, Moyer & Keppel, 1939); to decide this question, cystine was used as a supplement in an amount which would provide a sulphur content equivalent to that provided by methionine in the experiment in which the latter was used. The triethyl homologue of choline was included because it has been shown by Channon & Smith (1936) to possess lipotropic activity (though somewhat less than that of choline), and by McArthur, Lucas & Best (1947) to be biologically incorporated into the molecule of liver phospholipins. The high vitamin diet aimed at deciding whether some non-specific factor among the vitamins included in the diet was capable of influencing the activity of the enzyme when present at a higher level of intake.

In each experiment twenty male rats (150–180 g.) were used. Half were offered the basal diet, the other half received the same diet, with the supplement under investigation. The schedule was so arranged that each experiment was started 1 week following the initiation of the previous experiment. In each case the diets under consideration were offered for 4 weeks on a group-pair fed basis and the animals were killed in the usual manner.

In Table 5 a summary of the results obtained is presented. It will be seen that methionine, betaine and the triethyl homologue of choline, at the levels provided in their respective experiments, opposed the rise in plasma pseudo-cholinesterase observed when the diet is inadequate in its contents of lipotropic factors. The triethyl homologue of choline was not as efficient in this respect as the other two supple-

Table 6. *The effect of the ingestion of a hypolipotropic diet on the level of pseudo-cholinesterase in the plasma of adult female rats*

Group	Diet	Average total liver lipids (%, wet wt.)	Plasma pseudo-cholinesterase activity*		P
I	Basal	30·41	92·4 ± 9·9	2·74	$1·7 \times 10^{-2}$
II	Basal + choline chloride	5·49	135·3 ± 12·0†		

* Activity expressed as μl. CO_2 evolved in 20 min./ml. plasma.

† The level of the enzyme in the plasma of the animals receiving the choline-supplemented diet is higher than that of male rats on a similar diet (see Mundell, 1944).

ments; this may, however, be due to the fact that rats tend to refuse food in which this compound is included, and the level of intake of the substance may not have been optimal. The effect of methionine is not merely that of a sulphur-containing amino-acid because cystine not only fails to reduce the level of plasma pseudo-cholinesterase, but may promote a further elevation, though not in the present experiment a significant one. Its failure to prevent the rise in enzymic activity of the plasma agrees with the finding that cystine cannot replace methionine as a lipotropic agent. A fivefold increase in the vitamin content of the diet had no significant effect on the level of plasma pseudo-cholinesterase.

It would seem, therefore, that the ability of choline, methionine, betaine and the triethyl homologue of choline to prevent a rise in the pseudo-cholinesterase in the plasma of male rats is related to the activity of these compounds as lipotropic agents.

(*e*) *The effect of the ingestion of a hypolipotropic diet on the level of pseudo-cholinesterase in the plasma of adult female rats*

Twenty female rats (140–170 g.) were divided into two comparable groups. One group received the basal hypolipotropic ration the other, the same ration supplemented with choline chloride (0·35 %). The animals were group-pair fed for 4 weeks and then killed in the usual manner.

From Table 6, which contains the results of this experiment, it can be seen that the level of the pseudo-cholinesterase in female rat plasma does not rise when the intake of lipotropic factors is inadequate; instead, it shows a significant fall.

DISCUSSION

The rise in plasma pseudo-cholinesterase following the ingestion by male rats of a diet containing an inadequate amount of lipotropic agents is unexpected in view of the fact that the liver seems to be the main site of pseudo-cholinesterase synthesis and the accumulation of fat in that organ has been shown to be accompanied by an impairment of hepatic function. The level of this enzyme in the plasma of female rats shows, however, the expected decrease.

The difference in the response of male and female animals to a deficient intake of lipotropic agents suggests that the hypolipotropic diet is not directly responsible for the elevation observed in the males. Some, at the moment obscure, functional change may be induced in the males.

SUMMARY

1. Ingestion of a hypolipotropic diet leads to a highly significant rise in pseudo-cholinesterase activity in the plasma of adult and weanling male rats. The activity of true cholinesterase and of aliesterases in the plasma of these animals is unchanged.

2. The elevation in plasma pseudo-cholinesterase activity is accompanied by a significant rise in liver pseudo-cholinesterase activity.

3. Elevated levels of plasma pseudo-cholinesterase can be reduced to within the normal range by addition to the basal hypolipotropic diet of the lipotropic agents choline, betaine, methionine and the triethyl homologue of choline.

4. Female rats fail to show this elevated plasma pseudo-cholinesterase level when their diet is hypolipotropic.

5. A deficiency in lipotropic factors does not, therefore, seem to be the direct cause of the elevation in plasma pseudo-cholinesterase observed in male rats. The nature of the mechanism responsible for the elevation remains obscure.

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A Bridge Unit for Filter-paper Chromatography

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In chemical pathology filter-paper chromatography is becoming an analytical tool of increasing importance. In this particular field well established methods now allow for accurate identification of urinary sugars (Partridge, 1948), amino-acids (Dent, 1946) and porphyrins (Sveinsson, Rimington & Barnes, 1949).

In the present communication an apparatus is described that has been tested in this laboratory over the last nine months with satisfactory results. Some modifications in analytical technique are given here by way of example; others are the subject of further research.

Apparatus

The novel feature of this all-glass unit for filter-paper chromatography is the arrangement of the paper strip in the form of a bridge from one flask to another by passing it through a glass sleeve. All parts are provided with standard ground-glass joints, the whole unit being made to the author's specification by Quickfit and Quartz, Stone, Staffordshire.

The unit is easily assembled, and all parts are readily accessible for cleaning. It occupies little space on the laboratory bench, having an overall length of approximately 50 cm. The unit can be accommodated in the incubator or the refrigerator as the analytical procedure may demand, and since it is quite airtight it is highly suited for work with volatile solvents.

Fig. 1 shows the general appearance and mode of assembly of the bridge unit. The two 500 ml. flasks (*D*) form, as it were, the supporting pillars of the bridge. In practice, therefore, the glass bridge can be made to connect any one pair of flasks and no time need be lost in cleaning out the apparatus when changing from one method of analysis to another.

Parts *A* are T-shaped connecting pieces, which sit with their vertical limb on the flasks, the horizontal limb having one open and one slotted end (slot: 22 × 4 mm.); this latter end connects with part *C* or *B* of the bridge. The drawing of the assembly line of the bridge shows how parts *B* and *C* are arranged. Part *B* is a straight glass tube with cone and socket ends, the cone bearing a 9–10 mm. diameter hole in the ground-glass surface. Part *C* is also a glass tube, but here both ends form sockets and carry holes of the same size which locate with the holes in part *B*. One end of part *C* connects with part *A* to complete the bridge. The filter paper is introduced by opening the unit between parts *B* and *C* passing it either way through the slots into the flasks (Fig. 1), the paper just being held at small points of contact. When wet it sags, but even then it does not make contact with the glass along its broad surface.

The free ends of parts *A* are closed with caps *E* (standard size B34). These wide additional openings (internal diameter 28 mm.) to the unit were incorporated in the design for two reasons: first, they allow for the introduction of solutions and reagents to the unit after the filter-paper strip has been put in position and without disturbing it (a funnel with a semi-S-bent outlet is used for the purpose); secondly, this wide opening allows for the introduction of a pair of crucible tongs with which the filter-paper strip can be guided.

Since the holes in parts *B* and *C* are located in the ground-glass joints, the tubes may be rotated either to close or open them. In the former case ('closed' unit) the apparatus is

airtight and partition chromatography is carried out under these conditions. In the latter case ('open' unit) the holes are opened to allow evaporation. This procedure has been adopted in a novel form of chromatography in which the filter-paper strip forms a complete bridge between two

were selected as test material. The ordinary Whatman no. 1 filter paper commonly employed in this work did not seem quite suitable on account of its great thinness; a thicker paper was found in the fat-free strips of filter paper, Whatman no. 100, as used for milk-fat analysis. These strips measure

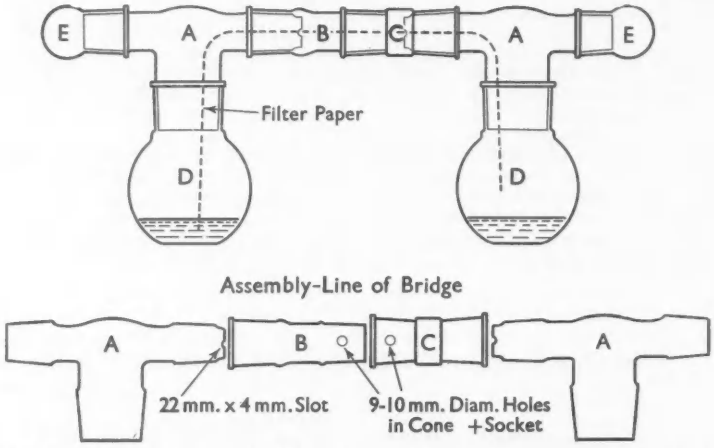

Fig. 1. Assembly of the bridge unit. (Note: the ventilators are not shown in the top drawing.)

different liquids, one of them being a solution of the substance to be analysed. The two liquids meet somewhere in the centre of the strip and continuation of flow is made possible by evaporation. Kawerau (1950) has given a preliminary report of chromatography of pathological urines carried out under these conditions, which will be published later in fuller detail.

Tests with the closed unit

In the first instance it was of importance to ascertain whether the change in the position of the filter paper affected R_F values to any significant extent. Carbohydrates

56 × 6 cm.; for use in the unit they are divided each into three strips of 2 cm. width. Washing by descending 'chromatography' was employed as it was found that distilled water removed small amounts of fluorescent and ninhydrin-reactive material, and, in agreement with the findings of Wynn (1949), the washed paper was also found to be faster.

R_F values were determined with A.R. samples of a number of sugars of pathological occurrence. The sugars were dissolved in distilled water or normal urine (0·25–1 mg./ml.) and 20 μl. of each solution were used for chromatography. The chromatogram was developed with the *n*-butanol-acetic acid-water system of Partridge & Westall (1948), the butanol

Table 1. *R_F values of some common carbohydrates* (n-*butanol-acetic acid-water* 4:1:5 *by vol.*)

(Values show the range observed; temperature was not controlled.)

Substance	Dissolving medium	R_F: With the closed bridge unit (Whatman no. 100 paper)	R_F: By either ascending or descending chromatography (see Horrocks & Manning, 1949)
D-Glucose	Urine	0·15–0·21	—
	Water	0·19–0·21	0·15–0·19
Lactose	Urine	0·04–0·08	—
	Water	0·05–0·07	0·05–0·09
D-Galactose	Water	0·19	0·12–0·17
D-Xylose	Urine	0·30	—
	Water	0·28–0·32	0·25–0·28
D-Galacturonic acid	Water	0·14–0·16	0·14
D-Arabinose	Water	0·18–0·24	0·21–0·22

layer being in the left-hand flask (Fig. 1), whilst the aqueous layer was in the right-hand flask. After chromatography, the strips were air dried and the sugar spots revealed with aniline hydrogen phthalate (Partridge, 1949). After removal from the oven the strips may be treated with a saturated solution of 'Perspex' polymethacrylate resin in chloroform. This serves to retard the development of background colour which would otherwise obscure the spots after a day or so. This treatment does not hinder the spots from fluorescing strongly in ultraviolet light. It does not, however, prevent ninhydrin-amino-acid spots from fading.

RESULTS

The results of thirty tests are summarized in Table 1, which gives the range of R_F values for each individual sugar. Comparison of the bridge-unit values with those recorded in the literature (Horrocks & Manning, 1949) show very close agreement, and the inference may be drawn that the modification in technique and the change in the position and nature of the filter-paper strip in no significant way affect R_F values.

SUMMARY

1. An all-glass bridge unit for filter-paper strip partition chromatography is described.

2. The unit can be used for any of the standard methods of one-dimensional partition chromatography; examples for carbohydrates are quoted.

3. The unit is supplied with ventilators, allowing a method of analysis in which continuous evaporation takes place from the paper.

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The Effect of Temperature on the Interference of Potassium in the Determination of Sodium

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In most of the microchemical methods for the determination of sodium in biological material the metal is precipitated as sodium zinc or magnesium uranyl acetate, and subsequently the uranium is estimated colorimetrically with potassium ferrocyanide or the acetic acid by titration. Phosphates are also precipitated by uranium salts, and must therefore first be removed with lime or ethanolic zinc sludge (McCance & Shipp, 1931*a*). Potassium as well as sodium forms a sparingly soluble zinc uranyl acetate. McCance & Shipp (1931*a*) considered that this would not interfere at 0°, provided the solution in which sodium was to be determined contained less than 0·3 mg. potassium/ml. Using the method of McCance & Shipp (1931*a*, *b*) for the determination of sodium in liver, unexpectedly high values were obtained. These could not be accounted for by sodium in the reagents, and the same high values were obtained however the phosphates had been removed. Since, moreover, no phosphate could be detected in the solutions after treating them with lime, it seemed improbable that phosphate was interfering.

As these high results were obtained both by colorimetry and titration, it was concluded that some potassium was being precipitated with sodium as the triple salt. Although the concentrations of potassium were well within the limits laid down by McCance & Shipp (1931*a*), the effect of temperature and time upon the precipitation of potassium was therefore reinvestigated using both acid extracts of liver tissue and pure solutions containing known amounts of sodium and potassium.

METHODS

For the precipitation and colorimetric determination of the triple salt, the method of McCance & Shipp (1931*a*) was used, with the following modifications. The precipitate of triple salt was dissolved in 5·0 ml. 10% (v/v) acetic acid, transferred to a 100 ml. flask and diluted almost to volume before adding 2·0 ml. 20% (w/v) $K_4Fe(CN)_6\ 3H_2O$. The optical density of the coloured, colloidal solution of uranium ferrocyanide was determined in the Spekker Absorptiometer using a 1 cm. cuvette and Ilford filter 604.

The amount of K present in the triple acetate precipitate was determined by dissolving the triple salt in water and

Table 1. *Effect of temperature on the precipitation of potassium with sodium as the zinc uranyl acetate triple salt*

Temperature (°)	Amounts taken Na (μg.)	Amounts taken K (μg.)	Apparent Na found (μg.)	K recovered (a) From precipitate (i) By calculation* (μg.)	K recovered (a) From precipitate (ii) By analysis (μg.)	K recovered (b) From supernatant by precipitation at −4° after Na had been removed (μg.)
−4	40	120	82	71	86	0
	100	300	230	219	243	0
0	40	120	67	49	—	—
	100	300	216	197	—	—
18	40	120	39	0	—	60
	100	300	100	0	—	170

* K was calculated from the increase in 'Na' content and expressed as μg. K.

removing K by precipitation with sodium cobaltinitrite (Eden, 1943), and then estimating the nitrite by the sulphonamide method of Bratton & Marshall (1939). (See Hawk, Oser & Summerson, 1947.)

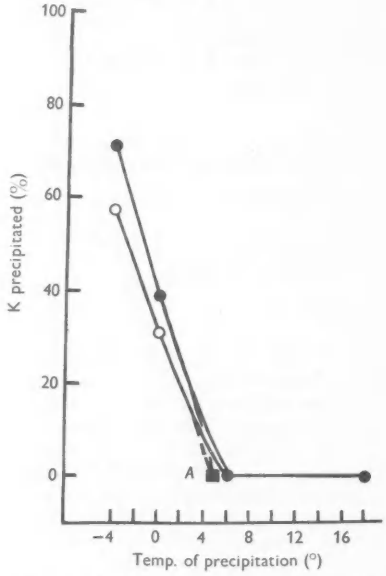

Fig. 1. The effect of temperature upon the precipitation of K: (*a*) from acid extract of liver tissue, ○—○; (*b*) from solution of NaCl and KCl of similar composition to (*a*), ●—●. ■, Point *A*, see text, p. 285.

For the determination of Na in liver tissue, the phosphate present in the acid extract of the ashed liver was removed with $Ca(OH)_2$. A sample containing 200–300 μg. Na was evaporated, and to it, when dry, were added 10·0 ml. water and sufficient $Ca(OH)_2$ (about 0·05 mg.) to make the solution alkaline to phenolphthalein. After 1 hr. it was filtered and the determinations were carried out on 2·0 ml. of the extract.

RESULTS

Effect of temperature. The results for the precipitation of potassium from solutions of sodium chloride and potassium chloride are summarized in Table 1.

These results show that, at 0 and −4°, the high values for sodium were caused by the simultaneous precipitation of potassium triple acetate. This was confirmed by the analyses of precipitates from solutions containing potassium alone.

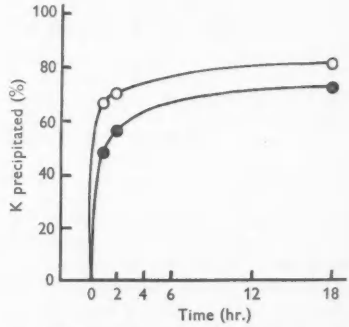

Fig. 2. The effect of time upon the precipitation of K from standard solutions of NaCl and KCl at −4° containing per 2·0 ml. sample: (*a*) 40 μg. Na and 120 μg. K, ●—●; (*b*) 100 μg. Na and 300 μg. K, ○—○.

Similar results were obtained for liver. Between 18 and 6°, the quantity of sodium found was always 2·87 m-equiv./100 g. wet tissue, but at 0 and −4° the sodium found increased to 5·83 and 8·17 m-equiv./100 g. wet tissue respectively. This interference of potassium is graphically represented in Fig. 1 where the temperature of the reactants is plotted against percentage potassium precipitated (*a*) from acid extract of ash of liver tissue, and (*b*) from standard solutions containing 40 μg. sodium and 120 μg. potassium/2·0 ml. sample taken for analysis which was equivalent to the sodium and potassium present

in the liver extract. Similar curves were obtained, and when the lines joining the points at 0 and $-4°$ were extrapolated, they converged and cut the x-axis between 4 and 6° (point marked A in Fig. 1).

By cooling solutions containing sodium and potassium, and potassium alone, it was also found experimentally that precipitation of potassium began within this range of temperature. The precipitation of potassium at $-4°$ was also demonstrated by warming to room temperature precipitates and supernatant solutions which had been at $-4°$ overnight. The potassium salt dissolved and the sodium 'found' in the tubes containing sodium and potassium was then equal to that found in the tubes containing sodium alone.

Effect of time. (*a*) Precipitation of potassium (Fig. 2).

In 1 hr. at $-4°$, 40–70 % of the potassium was precipitated, which caused an apparent increase of 70–115 % in the sodium. Within 2 hr. at the same temperature, over 50 % potassium was precipitated from the dilute solutions, and within 18 hr. 70–80 % potassium.

(*b*) Precipitation of sodium.

Sodium was precipitated quantitatively within 30 min. at room temperature (18°) from standard solutions of sodium and potassium, and from the liver extract.

DISCUSSION

These results show that under the conditions laid down by McCance & Shipp (1931*a*) some potassium was always precipitated with the sodium from a solution of the two ions. Salit (1932), also following the method of McCance & Shipp (1931*a*), observed an increase in precipitation of the triple salt at low temperatures but not at room temperature. Prof. R. A. McCance has informed me that in practice he and his collaborators always placed their tubes in a cool chamber, the temperature of which was always loosely referred to as '0°', but which was certainly not below 4°, or more recently in a modern refrigerator, set to maintain a temperature of 4°. There seems, therefore, no reason to suspect that any of the figures published by them are wrong, and a considerable number have been verified as correct within the last few months (McCance & Shipp, 1931*a*, *b*; McCance & Widdowson, 1940).

The present results confirm that precipitation of sodium is complete at room temperature (18°) (Peters & Van Slyke, 1932; Salit, 1932; Stone & Goldzieher, 1949), whereas McCance & Shipp (1931*a*) found precipitation incomplete at 20°. There is, however, little agreement between investigators about the exact formula of the crystalline precipitate of sodium triple acetate, some considering that it is $NaZn(UO_2)_3.(CH_3COO)_9.6H_2O$ (Barber & Kolthoff, 1928; Butler & Tuthill, 1931; Hald, 1933; Peters & Van Slyke 1932), and others

$$NaZn(UO_2)_3.(CH_3COO)_9.9H_2O$$

(Blanchetière, 1923). Perhaps both forms exist and have different solubilities. Hence different workers, using different media and techniques to precipitate the sodium triple salt, may be precipitating different crystalline forms of sodium triple acetate. The test of this hypothesis would require a complicated piece of research, and has not been attempted.

Quantitative precipitation of potassium has not been observed at the temperatures employed in this investigation. It might be possible, however, to separate the two cations and determine them both with the same reagents by precipitating the sodium at room temperature, and subsequently the potassium at a much lower one.

SUMMARY

1. When sodium is determined in the presence of potassium, precipitation of the triple acetate should be carried out above 4–6°. Below this temperature potassium is precipitated, and at 0° and $-4°$ the apparent sodium content of liver tissue increases to two and three times the value at 6° respectively.

2. Between 6 and 18° there is quantitative recovery of sodium in 30 min.

I wish to thank Prof. R. A. McCance for his advice and assistance throughout this investigation.

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The Assimilation of Amino-acids by Bacteria

10. ACTION OF INHIBITORS ON THE ACCUMULATION OF FREE GLUTAMIC ACID IN *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS FAECALIS*

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Certain Gram-positive bacteria have the ability to concentrate certain amino-acids in the free state within the cell (Gale, 1947; Taylor, 1947). Amino-acids may pass across the cell wall either by a process of diffusion, as in the case of lysine which becomes concentrated within the cell by a form of Donnan effect (Najjar & Gale, 1950), or as a result of an active transfer which requires energy obtained from exergonic metabolism such as fermentation. Glutamic acid does not enter either *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) or *Streptococcus faecalis* unless these organisms are metabolizing glucose, but the mechanism of this 'energy-linked' transfer across the cell wall is not yet known. Gale & Mitchell (1947) showed that the concentration of the free amino-acid within the cell is determined by the balance between the rate of passage into the cell and the rate of metabolism within the cell; thus a substance such as crystal violet which inhibits the internal metabolism of glutamic acid in *Strep. faecalis* increases the steady state concentration of free glutamic acid within the cell, while treatment of the cell with penicillin (Gale & Taylor, 1947) inhibits the passage of glutamic acid into *Staph. aureus* so that the internal concentration decreases until exhausted by internal metabolic processes. The passage of glutamic acid into *Staph. aureus* is also inhibited by 8-hydroxyquinoline which appears to act by chelation of a metal essential to the process; deprivation experiments show that the transfer is activated by manganese, although this can be replaced by magnesium in higher concentration (Gale, 1949).

Clifton (1946) has shown that oxidative assimilation of carbon substrates in Gram-negative organisms is inhibited by sodium azide or 2:4-dinitrophenol. These, together with arsenate, appear to act by uncoupling the generation of energy-rich phosphate bonds from oxidative processes (Needham & Pillai, 1937; Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949). Spiegelman, Kamen & Sussman (1948) have shown that sodium azide prevents the transfer of inorganic phosphate to the organic fraction accompanying the coupled oxidation of glyceraldehyde phosphate. The present communication reports the action of these inhibitors on the accumulation of free glutamic acid within *Staph. aureus* and *Strep. faecalis*. During the course of the work it became clear that the process involved in the passage of glutamic acid into *Staph. aureus* has properties and sensitivities to inhibitors different from those of the similar process in *Strep. faecalis*. This can be correlated with differences previously noted in the quantitative aspect of the internal metabolism of glutamic acid (Gale & Rodwell, 1949) and in the conditions controlling the diffusion of free glutamic acid out of the cells (Gale, 1948).

METHODS

Organisms. Three organisms were used in the course of this work: *Staph. aureus* Duncan (Gale & Taylor, 1947); *Strep. faecalis* ST (National Collection of Type Cultures (N.C.T.C.) no. 6782) and *Strep. faecalis* Dunn (N.C.T.C. no. 6783).

Growth media. The organisms were grown for 16 hr. at 30° in the 'deficient' medium B previously described (Gale, 1947). This consists of a salt medium containing 1% glucose, 0·1% marmite and 0·1% (w/v) arginine; for the growth of *Strep. faecalis* strains pyridoxin, riboflavin and pantothenic acid were also added. The medium was made up in flasks for *Strep. faecalis*, and in 150 ml. quantities in Roux bottles to give good aeration for the growth of *Staph. aureus*. The cells were harvested on the centrifuge and washed once with distilled water. Suspensions were prepared containing approx. 20 mg. dry wt. of cells/ml. in water and these were diluted 1:10 in the reaction solutions for the experiments described below.

Estimation of glutamic acid and its accumulation within the cells. Free glutamic acid was estimated manometrically with glutamic acid decarboxylase preparations (Gale, 1945). The accumulation of free glutamic acid by the washed cells was studied as previously described (Gale, 1947) by estimation of the glutamic acid content of cell suspensions before and after rupture of the cells by boiling.

RESULTS

Preliminary investigations. Investigations of the inhibitory action of sodium azide on the passage of glutamic acid into bacterial cells were first carried out in parallel on *Staph. aureus* and *Strep. faecalis* strains. It was then found that concentrations of sodium azide which caused an inhibition of the

passage of glutamic acid into *Staph. aureus* produced a marked increase in the amount of this amino-acid which accumulated under similar experimental conditions inside *Strep. faecalis*. It became clear that there are at least two processes, first, the passage of glutamic acid from outside to the inside of the cell and, secondly, the metabolism inside the cell after the first process has taken place. It seemed that sodium azide affects the first of these two processes in *Staph. aureus* whereas, in *Strep. faecalis*, the second process is more sensitive to the action of azide than the first; consequently, an enhanced accumulation of free glutamic acid is found in the latter case but not in the former. Later investigations showed that many of the inhibitors studied have this differential action. Consequently, it is necessary to present separately the results obtained with the two organisms.

Inhibition of the accumulation of glutamic acid in Staphylococcus aureus

Sodium azide and 2:4-dinitrophenol. Fig. 1 shows the degree of inhibition of the rate of accumulation of free glutamic acid within *Staph. aureus*. The passage

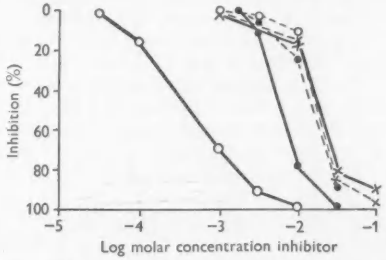

Fig. 1. Effect of inhibitors on glutamate accumulation and glucose fermentation in *Staph. aureus*. Organisms grown in medium B (Gale, 1947); prepared in washed suspension and incubated at 37° in buffered salt solution containing 1% glucose and 10 μmol. sodium glutamate/ml. Free glutamate within cells determined at intervals and rate of accumulation determined with and without inhibitors. Fermentation investigated in Warburg manometers containing 1·0 ml. washed suspension (3–4 mg. dry wt./ml.), 1·0 ml. 0·03 M-$NaHCO_3$, 0·5 ml. 0·05 M-glucose and 0·5 ml. water or inhibitor solution; manometers gassed with N_2 containing 5% CO_2. ——, glutamate accumulation; – – – –, glucose fermentation. ○, 2:4-dinitrophenol; ●, sodium azide; ×, sodium fluoride.

of this amino-acid into the cell is an energy-linked reaction and only occurs, under the experimental conditions, when fermentation of glucose occurs as source of energy. The figure also shows the inhibition of the rate of glucose fermentation, and it can be seen that the passage of glutamate into the cell is markedly more sensitive to inhibition by sodium azide and 2:4-dinitrophenol than the energy-providing fermentation system. In the case of sodium azide, the concentration producing 50% inhibition is 0·006 M for the passage of glutamate and 0·014 M for fermentation; the corresponding concentrations for 2:4-dinitrophenol are 0·0004 and 0·02 M. The degree of inhibition varies with the pH of the reaction mixture; Fig. 2 shows that the sensitivity of the glutamate-transferring system to dinitrophenol increases 300 times if the pH falls from the usual experimental value of 7·0 to 5·0.

Fig. 1 also shows results obtained with sodium fluoride as inhibitor. In this case the accumulation of free glutamate is inhibited to the same extent as the fermentation system, suggesting that impairment of energy provision causes an equivalent inhibition of glutamate transfer. Other inhibitors, whose action is like that of fluoride in this respect, are iodoacetic acid and patulin.

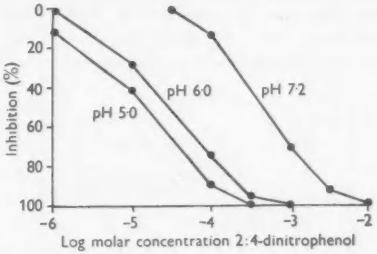

Fig. 2. Effect of pH on the inhibition of glutamate accumulation in *Staph. aureus* by 2:4-dinitrophenol. Conditions as for Fig. 1 with adjustment of buffered salt solution to pH values shown.

Sodium arsenate. The effect of sodium arsenate on the rates of glycolysis and of glutamate accumulation in *Staph. aureus* is shown in Table 1. 0·0001 M-Sodium arsenate causes 60% increase in the rate of fermentation, although concentrations higher than 0·01 M are inhibitory. The rate of glutamate accumulation is also increased by the presence of arsenate, although the highest increase recorded is 26% at a concentration of 0·001 M and no inhibitory action was found at the highest concentration (0·03 M) tested.

Effect of inhibitors on the accumulation of glutamic acid by Streptococcus faecalis

Sodium azide. If washed cells of *Strep. faecalis*, rendered deficient in free glutamic acid by growth in an amino-acid deficient medium (Gale, 1947), are incubated for 1 hr. in a buffered salt solution containing 10 μmol. sodium glutamate/ml. and glucose (1·0%) with and without 0·01 M-sodium azide, it is found that the content of free glutamic acid at the end of the incubation may be 300–700% higher in the cells treated in the presence of azide than in those

Table 1. *Effect of sodium arsenate on fermentation and glutamic acid accumulation in* Staphylococcus aureus

(Organism grown for 16 hr. at 30° in medium B (Gale, 1947). Accumulation of internal free glutamate determined before and after incubation for 45 min. at 37° in buffered salt solution containing 1% glucose and 10 μmol. sodium glutamate/ml. Results expressed as μmol. increase in free glutamate/hr./100 mg. dry wt. of cells. Fermentation determined in manometers containing 1·0 ml. washed suspension (approx. 3 mg. dry wt. of cells/ml.), 1·0 ml. 0·03 M-$NaHCO_3$, 0·5 ml. 0·05 M-glucose and 0·5 ml. water or sodium arsenate solution; manometers filled with N_2 containing 5% CO_2.)

Concentration of sodium arsenate (mM)	Fermentation		Accumulation of free glutamate	
	Q_{CO_2} (μl./mg. dry wt./hr.)	Effect (%)	μmol. glutamate/hr./100 mg. dry wt. cells	Effect (%)
0	113	—	34·0	—
0·1	184	+62	34·2	0
1·0	175	+54	42·9	+26
3·0	144	+27	38·6	+14
10·0	105	− 7	36·9	+ 8
30·0	48	−49	36·0	+ 6

treated in its absence. Fig. 3 shows the rate of appearance of free glutamic acid in the cells under these conditions; it can be seen that azide has increased the rate at which the free glutamate

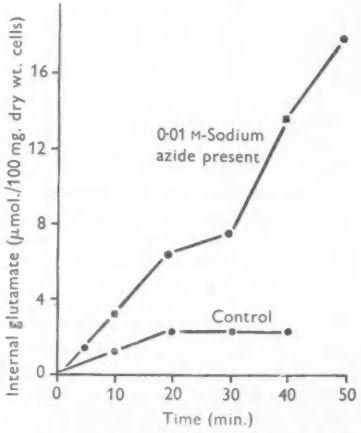

Fig. 3. Effect of sodium azide on the accumulation of free glutamate within *Strep. faecalis* cells. Deficient cells incubated at 37° in buffered salt solution containing 1% glucose and 10 μmol. sodium glutamate/ml. with and without 0·01 M-sodium azide. Cells removed at intervals, rapidly cooled, washed and internal free glutamate determined.

accumulates and also the final internal concentration attained. Fig. 4 shows the effect of the concentration of sodium azide on the level attained after 1 hr. incubation, in comparison with the level attained in control cells without azide; there is apparently a double response. At low concentrations, the presence of azide results in a lowering of the concentration of glutamate below that attained in the control; at high concentrations, the presence of azide produces an internal concentration of glutamic acid markedly greater than that attained in the control. Parallel experiments on the rate of fermentation show that concentrations of azide which produce a fall in the internal glutamate concentration produce a significant acceleration of fermentation.

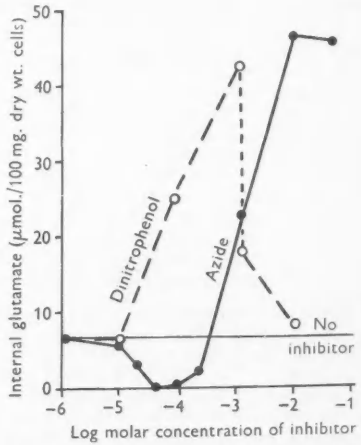

Fig. 4. Effect of 2:4-dinitrophenol and sodium azide on the accumulation of free glutamate in *Strep. faecalis*. Conditions as for Fig. 3; samples taken at end of 45 min. at 37° for determination of internal free glutamate.

Gale & Mitchell (1947) showed that the increase in the internal concentration of free glutamic acid in *Strep. faecalis*, produced by the presence of crystal violet and other dyes of the triphenylmethane series, was due to an inhibition of the internal metabolism of glutamic acid within the cells. Experiments have been carried out with sodium azide similar to those reported with crystal violet, and Table 2 shows that the same inhibition of internal metabolism can

Table 2. *Internal metabolism of glutamic acid in* Streptococcus faecalis

(*Strep. faecalis* grown in deficient medium, washed and incubated at final suspension density of 1–2 mg. dry wt. of cells/ml. in buffered salt solution containing 1% glucose and approx. 70 μmol. sodium glutamate. After 1 hr. at 37°, cells removed and free glutamate content determined. Content of cells and supernatant before and after incubation determined and net loss of glutamic acid calculated = glutamic acid metabolized (Gale & Mitchell, 1947).)

Inhibitor	Concn. (mM)	Glutamate metabolized/100 mg. dry wt. cells: μmol.		Metabolism in control (%)
None	—	14·9, 15·0, 12·5, 13·0	13·8	100
Sodium azide	10·0	1·3, 2·3, 1·6, 3·4	2·15	15·6
	0·1	19·9		144
2:4-Dinitrophenol	0·1	10·0		70

be demonstrated with 0·01 M-sodium azide. 0·0001 M-Sodium azide, which produces a decrease in the internal concentration of glutamate, accelerates the disappearance of free glutamate from within the cells. No increase in internal free glutamate can be shown in cells incubated with or without azide in the absence of external glutamate, so the increased concentration of internal free glutamate found in some cases cannot be due to any autolytic process. Consequently, it is probable that the variations in the internal level in the presence of azide can be explained, as previously postulated (Gale & Mitchell, 1947), by variations in the rate of internal metabolism of the free glutamic acid.

2:4-*Dinitrophenol.* The addition of dinitrophenol to the external medium during the accumulation of glutamic acid in the presence of glucose by *Strep. faecalis* produces an increase in the internal free glutamate concentration attained (Fig. 4). At concentrations greater than 0·001 M, dinitrophenol appears to inhibit the passage of the amino-acid into the cell so that the internal concentration again falls. The concentration 0·001 M is critical; in some cases it produces an increase in the internal concentration to a value equal to that attained in the presence of 0·01 M-sodium azide, but in other experiments under similar conditions the effect is much less marked, suggesting that partial inhibition of the passage of the amino-acid into the cell is taking place. Table 2 shows that 0·0001 M-dinitrophenol, which gives an increase in internal glutamate concentration equal to about 50% of that produced by 0·01 M-sodium azide, also effects approximately 70% inhibition of the internal metabolism of glutamic acid.

Sodium arsenate. 0·0001 M-Sodium arsenate produces 25% increase in the internal concentration of glutamate in *Strep. faecalis*; higher concentrations inhibit the appearance of free glutamate within the cells.

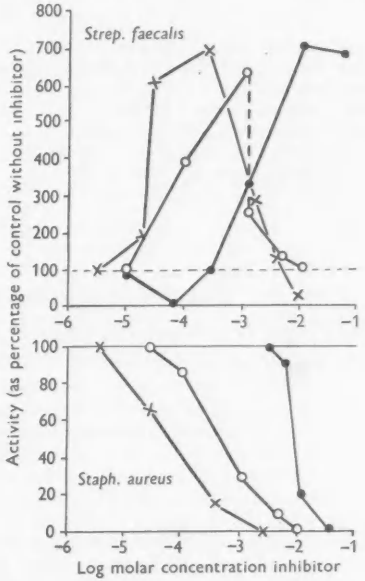

Fig. 5. Effect of inhibitors on the accumulation of glutamate in *Staph. aureus* and *Strep. faecalis*. Conditions as for Figs. 1 and 4. ×—×, crystal violet; ○—○, 2:4-dinitrophenol; ●—●, sodium azide.

Comparison of effects of inhibitors on Staphylococcus aureus *and* Streptococcus faecalis. Fig. 5

shows the actions of sodium azide, dinitrophenol and crystal violet on the accumulation of free glutamate in *Staph. aureus* and *Strep. faecalis* exposed to glutamate and glucose. The three substances produce an inhibition of the passage of the amino-acid into *Staph. aureus* but an increase in the internal concentration attained in *Strep. faecalis* although, in high concentrations, they appear to have an inhibitory action on the passage of the amino-acid into the cells in this case as well. The latter inhibition can, in the case of crystal violet, be correlated with interference with the energy-supplying fermentation system in *Strep. faecalis* (Gale & Mitchell, 1947), although this is not the explanation for the inhibition of the passage into *Staph. aureus*. The increase in the internal concentration of free glutamate in *Strep. faecalis* can be ascribed to an inhibition of metabolism of the amino-acid within the cell, and it is interesting to note that concentrations of the three substances which produce optimal inhibition of internal metabolism in *Strep. faecalis* are approximately the same as those producing almost complete inhibition of passage into *Staph. aureus*.

SUMMARY

1. The accumulation of free glutamic acid within *Staphylococcus aureus* requires energy supplied, under the experimental conditions used, by the fermentation of glucose. Sodium azide and 2:4-dinitrophenol inhibit the accumulation of free glutamate without inhibiting the fermentation reactions.

2. Sodium arsenate accelerates the rates of fermentation and of glutamate accumulation.

3. Sodium azide and 2:4-dinitrophenol increase the amount of free glutamate accumulating within *Streptococcus faecalis* incubated in the presence of glutamic acid and glucose. The effect can be correlated with the inhibition, by these substances, of internal metabolism of glutamic acid in these cells.

4. Concentrations of sodium azide and 2:4-dinitrophenol producing optimal increase in the internal glutamate concentration in *Strep. faecalis* are approximately the same as those producing complete inhibition of glutamate accumulation in *Staph. aureus*.

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The Assimilation of Amino-acids by Bacteria

11. THE RELATIONSHIP BETWEEN ACCUMULATION OF FREE GLUTAMIC ACID AND THE FORMATION OF COMBINED GLUTAMIC ACID IN *STAPHYLOCOCCUS AUREUS*

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Staphylococcus aureus (*Micrococcus pyogenes* var. *aureus*), in common with certain other Gram-positive bacteria, possesses the ability to concentrate certain amino-acids in the internal environment (Gale, 1947*a*, *b*; Taylor, 1947). In the case of glutamic acid, passage into the cell only occurs when some exergonic metabolism such as glycolysis is also taking place. If cells containing little free glutamic acid are incubated in a medium consisting of buffered saline, glucose and glutamic acid, the amino-acid enters the cell until the internal concentration is many times that in the external medium. The passage of the amino-acid into the cell is prevented by any substance which inhibits glycolysis and is also inhibited, at concentrations ineffective against glycolysis, by sodium azide, 2:4-dinitrophenol and 8-hydroxyquinoline (Gale, 1949, 1951). All the studies so far reported on the passage of glutamic acid into *Staph. aureus* and other Gram-positive cells have referred to the process which takes

place when 'deficient' cells are incubated in a simple buffered salt and glucose medium with glutamic acid as the only amino-acid present. Under these conditions the passage into the cell and accumulation therein of free glutamic acid are accompanied by metabolism of the glutamic acid but no glutamyl-peptides or related substances are formed within the cell (Gale & Mitchell, 1947). Hotchkiss (1947) has reported that washed suspensions of *Staph. aureus* incubated with a mixture of amino-acids and glucose assimilate amino-acids and synthesize protein without cellular proliferation. It seemed possible, therefore, that the addition of other amino-acids to the usual reaction mixture used in these studies would lead to protein synthesis and enable the relationship, which exists between the accumulation of free glutamic acid within the cell, and the condensation of glutamic acid into cell protein, to be determined. Previous studies (Gale, 1947*b*) on the concentration of free glutamic acid within growing cells suggested that condensation into protein followed the concentration within the cell of free amino-acids, but the evidence was indirect. In the experiments reported here, the free glutamic acid content of the cells and the combined glutamic acid liberated by acid hydrolysis have been estimated; the combined glutamate fraction will therefore include the glutamic acid of cell proteins and peptide structures within the cell.

METHODS

Organisms. Staphylococcus aureus Duncan (Gale & Taylor 1947) was used throughout the work described in this paper.

Growth medium. The organism was grown for 16 hr. at 30° in the 'deficient' medium previously described (Gale, 1947*a*, 1951). The cells were harvested on the centrifuge and washed once with distilled water. Suspensions were prepared containing approximately 20 mg. dry wt. of cells/ml. and these were diluted 1:10 in the reaction solutions for the experiments described below.

Estimation of glutamic acid and its accumulation by Staphylococcus aureus. Free glutamic acid was estimated manometrically with glutamic acid decarboxylase preparations (Gale, 1945). The accumulation of free glutamic acid by the washed cells was studied as previously described (Gale, 1947*a*).

Combined glutamic acid. For studies of the formation of combined glutamic acid, a mixture of amino-acids, other than glutamic acid, was made up to contain 2·0 mg./ml. of the natural isomers of each of the following: (*A*) aspartic acid, tyrosine, tryptophan, phenylalanine, asparagine; (*B*) histidine, lysine, arginine; (*C*) proline, serine, alanine, threonine, methionine; (*D*) leucine, isoleucine, cystine, glycine, valine. In some experiments the mixtures *A*, *B*, *C* and *D* were made up separately. Vitamin-free casein hydrolysate was prepared by acid hydrolysis of vitamin-free casein (Glaxo Laboratories) followed by treatment of the hydrolysate with activated charcoal. All amino-acid mixtures were adjusted to pH 7·0 before use.

The total glutamic acid content of cell suspensions was estimated after hydrolysis of the cells in boiling 5 N-HCl for 18 hr. The combined glutamate was then calculated from the difference between total and free glutamic acid content. Supernatant solutions obtained after centrifuging down the cells at the end of the experiments were also examined for the presence of combined glutamate in a similar manner.

RESULTS

Preliminary work. During some studies on the effect of the constituents of the growth medium on the ability of washed *Staph. aureus* to accumulate free glutamic acid, it was found that the rate of accumulation was reduced by approximately 85 % in the presence of 1 % marmite solution. This effect could be reproduced by substitution of the marmite solution by hydrolysed marmite, casein digest, vitamin-free casein hydrolysate or a mixture of amino-acids. Estimation of the combined glutamate of the cells showed that this increased during incubation in the presence of such amino-acid mixtures and glucose, but that no increase occurred during incubation with glutamic acid and glucose alone. The concentration of free glutamic acid within the metabolizing cell has been previously shown (Gale & Mitchell, 1947) to be determined by the balance between the rate at which it enters the cell and the rate of internal metabolism. Consequently it seemed probable that the decreased rate of accumulation of free glutamic acid when other amino-acids were present was due to an increased rate of internal metabolism consequent upon the glutamic acid entering into combination with other amino-acids. If the rate of entry of glutamic acid into the cell were unaffected by the presence of other amino-acids, and combination with other amino-acids occurred after glutamic acid had accumulated in the free state within the cell, then the rate of uptake of glutamic acid from the external medium should be approximately the same, whether the mixture of other amino-acids were present or not. Experimental test showed that this was not the case but that the rate of uptake of glutamic acid was markedly decreased by the presence of amino-acid mixtures giving rise to the formation of combined glutamate within the cells.

Effect of casein hydrolysate on the accumulation of free glutamic acid. Fig. 1 shows the accumulation of free glutamic acid within *Staph. aureus* when the cells are incubated in buffered saline containing glucose and glutamic acid with and without the addition of vitamin-free casein hydrolysate. The rate of accumulation is almost nil in the presence of 0·7 % casein hydrolysate, and Table 1 shows that an increase in the combined glutamate of the cells is occurring in the presence of the hydrolysate but not in its absence. Such an increase in the combined glutamate of the cells occurs when a mixture of many amino-acids is present, and it seemed probable that restricting the amount of hydrolysate would limit

the duration of the combining process. Fig. 1 shows that the addition of 0·08 or 0·16 % casein hydrolysate causes a break in the course of free glutamic acid accumulation. It is not possible to carry out accurate detailed analyses over the short periods covered by these breaks, but estimations show that an increase in the combined glutamate of the cells occurs during the periods covered by the breaks. In the presence of these low concentrations of casein hydrolysate, the accumulation of free glutamic acid starts at approximately the normal rate; it then almost ceases during the period when combined glutamate is being formed, and is resumed at the normal rate again when combination ceases.

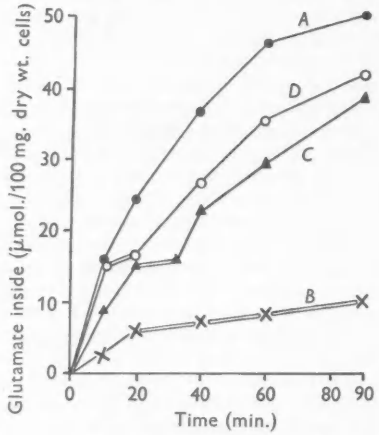

Fig. 1. Rate of accumulation of free glutamic acid in *Staphylococcus aureus* incubated with glucose, glutamic acid and casein hydrolysate. Deficient cells incubated at 37° in buffered salt solution containing 1 % glucose, 10 μmol. sodium glutamate/ml. and casein hydrolysate as indicated below. Samples taken at intervals, rapidly cooled, washed and the free internal glutamate determined. Final concentration of casein hydrolysate added: *A*, none; *B*, 0·7 %; *C*, 0·16 %; *D*, 0·08 %.

Table 1 gives a detailed analysis of the changes in free and combined glutamate of the medium and cells occurring during 1 hr. incubation under conditions corresponding to those of curves *A* and *B* of Fig. 1. In the absence of casein hydrolysate, free glutamic acid accumulates in the cells and approximately twice the amount that accumulates is removed from the external medium; there is no significant increase in the combined glutamate of cells or medium. In the presence of casein hydrolysate, the amount of free glutamic acid accumulating within the cells is equal to 9 % of that accumulating in the absence of hydrolysate, and the amount of glutamate withdrawn from the medium lies within the experimental error of the estimations. The amount of glutamic acid added in the casein hydrolysate was so great that accurate estimation of the changes in the medium was not possible; attempts were therefore made to add glutamate-free casein hydrolysate by prior treatment of the hydrolysate with glutamic acid decarboxylase preparations. However, when complete glutamic acid balances were attempted, including determinations of any combined glutamate in the medium before and after incubation, it was found that treatment with the decarboxylase preparation frequently resulted in the addition of protein to the hydrolysate and that this addition could neither be controlled nor standardized. Some results obtained with this material are quoted in Table 4, but it was decided that the hydrolysate must be replaced by a synthetic mixture of amino-acids.

Effect of amino-acid mixtures on the accumulation of free glutamic acid. Table 2 shows the effect on the accumulation of free glutamic acid within the cells of adding various mixtures of amino-acids to the glucose, glutamate and salt solution comprising the external

Table 1. *Changes in glutamic acid distribution during incubation of* Staphylococcus aureus *in the presence of glutamic acid and glucose with and without casein hydrolysate*

(Cells grown for 16 hr. at 30° in deficient medium, harvested and made into washed suspension. Incubated at final suspension density = approx. 2 mg. dry wt. cells/ml. in buffered salt solution containing 1 % glucose, 1·34 μmol. sodium glutamate/ml. and 1·0 % vitamin-free casein hydrolysate. Free and combined glutamate of cells and supernatant determined before and after 1 hr. at 37°; results expressed as change (μmol.) in glutamate/100 mg. dry wt. of cells.)

Incubation mixture ...	Glucose (*a*)	Glucose + glutamate (*b*)	Glucose + glutamate + casein hydrolysate (*c*)	Initial value
Internal free glutamate of cells	−4·46	+34·2	+2·9	19·0
External free glutamate of medium	+2·4	−63·0	−1·8*	(*b*) 72·0 (*c*) 398·0
Combined glutamate of cells	0	+ 0·13	+5·7	36·8
Combined glutamate of medium	0	0	0	0
Balance	−2·06	−28·6	+6·8*	

* Query experimental error owing to large amount of free glutamate in mixture (*c*).

Table 2. *Effect of presence of amino-acids on accumulation of free glutamic acid*

(*Staph. aureus* Duncan grown for 16 hr. at 30° in deficient medium, cells harvested and made into washed suspension; incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, 9 μmol. sodium glutamate/ml. with or without the addition of various mixtures of amino-acids, as below, each at a final concentration of 0·2 mg. L-isomer/ml.)

Amino-acids present (other than glutamic acid)	Increase in internal free glutamic acid (μmol./100 mg. dry wt. cells)	Reduction in rate of free glutamate accumulation (%)
None	32·6	—
Mixture *A* (aspartic; tyrosine, tryptophan, phenylalanine, asparagine)	16·4	50
Mixture *B* (histidine, lysine, arginine, methionine)	24·3	25
Mixture *C* (proline, serine, alanine, threonine)	16·7	48
Mixture *D* (leucine, cystine, isoleucine, glycine, valine)	15·8	52
Mixtures *A* + *B*	12·1	63
Mixtures *A* + *B* + *C*	6·9	79
Mixtures *A* + *B* + *C* + *D*	0·27	92

Table 3. *Changes in glutamic acid distribution during incubation of* Staphylococcus aureus *in the presence of glutamic acid and glucose with and without the addition of a mixture of eighteen other amino-acids*

(Conditions as for Table 1 with substitution of the amino-acid mixture (final concentration of each amino-acid = 0·2 mg./ml.) for casein hydrolysate. Results expressed as change (μmol.) in glutamate/100 mg. dry weight of cells.)

Incubation mixture ...	Glucose	Glucose + glutamate	Glucose + glutamate + amino-acid mixture	Glutamate + amino-acid mixture	Initial value
Internal free glutamate of cells	+0·2	+29·8	− 6·9	− 1·5	14·1
External free glutamate of medium	+1·48	−48·0	− 7·5	+11·4	69·0
Combined glutamate of cells	−0·56	− 0·2	+11·2	−13·4	32·5
Combined glutamate of medium	+0·9	+ 1·3	0	0	0
Balance	+2·02	−17·1	− 3·4	− 3·5	115·6

Table 4. *Changes in glutamic acid distribution during incubation of* Staphylococcus aureus *in the presence of glutamic acid and glucose with and without a source of other amino-acids*

(Conditions as for Tables 1 and 3. Control = glucose, glutamate, buffered salt solution.)

	Glutamate removed from medium		Change in internal free glutamate		Increase in combined glutamate		Loss of glutamate on balance	
Dry wt. cells (mg.)	Control (μmol.)	Amino-acids added (μmol.)	Control (μmol.)	Amino-acids added (μmol.)	Control (μmol.)	Amino-acids added (μmol.)	Control (μmol.)	Amino-acids added (μmol.)
A. Casein hydrolysate as amino-acid source								
123	63·0	1·8	+34·2	+ 2·9	0	5·7	17·2	3·45
98	44·1	9·8	+34·2	+ 2·0	0	11·1	17·8	—
112	35·5	15·6	+24·1	− 3·3	0	10·0	10·7	—
87	46·9	12·5	+32·9	+ 6·1	0	13·0	6·7	—
B. Complete amino-acid mixture as amino-acid source								
106	48·0	7·4	+29·8	− 6·9	0	11·2	17·2	3·45
116	56·8	5·35	+32·2	− 5·8	0	6·35	28·4	4·8
100	55·1	8·5	+30·1	−10·2	0	6·7	27·5	7·18
103	54·9	6·95	+30·5	− 8·05	0	8·15	31·0	8·6
111	55·0	0·7	+33·8	−12·3	0	6·9	25·7	4·7

medium. The glutamic acid concentration in the external medium was kept constant at 10 μmol./ml. The addition of any of the mixtures resulted in a reduction of the rate of accumulation of free glutamic acid. Increasing the total number of amino-acids added resulted in progressive inhibition, and addition of the complete mixture of nineteen amino-acids abolished the accumulation of free glutamic acid almost completely. In later experiments the concentration of glutamic acid in the medium was reduced to 1·3 μmol./ml. and the addition of the mixture of eighteen amino-acids, each at a final concentration of 0·2 mg./ml., then resulted in a decrease in the internal glutamic acid concentration during incubation. The suppression of the rate of accumulation of the free amino-acid within the cells can be correlated with the completeness of the external amino-acid mixture regarded as protein precursor. A similar effect of the quality of the amino-acid mixture has been noted by Pollock & Wainwright (1948) in their studies of the stimulation of nitratase formation by washed organisms. Table 3 shows that the cells form combined glutamate when incubated with the complete amino-acid mixture. The incomplete amino-acid mixtures, which effect a partial suppression of the accumulation of free glutamic acid, do not necessarily promote the formation of combined glutamate within the cells, and their effects will be dealt with in a later communication.

Table 3 gives a detailed analysis of the changes occurring in the distribution of glutamic acid during the incubation of *Staph. aureus* with various components of the glucose, glutamate and amino-acid mixture system. No increase in combined glutamate of the cells occurs in the absence of the amino-acid mixture or glucose. When the cells are incubated with glucose and glutamic acid alone, a marked accumulation of free glutamic acid occurs within the cells and more glutamic acid is taken up from the medium than appears in the free state within the cells. In no case is there any significant appearance of combined glutamate in the supernatant medium, the small amounts recorded in some experiments probably being due to autolysis of some of the cells during incubation. The discrepancy between the amount of glutamic acid removed from the medium and the amount appearing as the free amino-acid within the cells corresponds to the metabolism which takes place under these conditions (Gale & Mitchell, 1947). When the amino-acid mixture is added to the external medium, the amount of glutamate removed from the medium within a given time is greatly reduced, the concentration of free glutamic acid within the cells falls, the combined glutamate of the cells increases, and the amount of glutamic acid unaccounted for in the balance sheet is greatly reduced. Table 4 summarizes several experiments, carried out with the complete synthetic amino-acid mixture or vitamin-free casein hydrolysate as external source of amino-acids, which support these conclusions.

Effect of internal concentration of free glutamic acid on rate of formation of combined glutamate

In the experiments described above, the formation of combined glutamate in the cells was accompanied by a decrease in the concentration of internal free glutamic acid. In order to determine whether

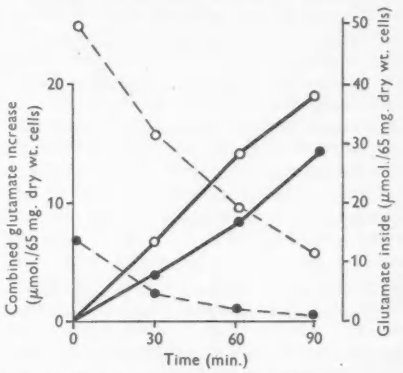

Fig. 2. Relation between internal free glutamate concentration and rate of formation of combined glutamate in *Staphylococcus aureus*. Deficient cells treated either with glucose and glutamate (○) or with glucose alone (●) and then incubated in buffered salt medium containing 1% glucose, and a mixture of 18 other amino-acids (final concn. 0·2 mg. each amino-acid). Samples taken at intervals and internal free glutamate determined (– – –); cells then hydrolysed with boiling 5 N-HCl for 18 hr. and combined glutamate determined. ——, increase in combined glutamate of cells.

the rate of combination of glutamate is affected in any way by the initial concentration of free glutamic acid within the cells, experiments were carried out in which the suspension of *Staph. aureus* was divided into two portions, one of which was first incubated in buffered salt solution containing glucose and glutamic acid, while the other was incubated in a similar solution without glutamic acid; the two suspensions were then washed, incubated with the complete amino-acid mixture (less glutamic acid) and glucose, and their rates of formation of combined glutamate compared. Table 5 gives a detailed analysis of the glutamic acid changes in such an experiment, and it can be seen that the cells which had been previously saturated with internal free glutamic acid form combined glutamate at a rate approximately 340% greater than that attained in the cells with low internal free glutamic acid. The concentration of free glutamic acid within the pretreated cells falls very rapidly on incubation with the amino-acid mixture

Table 5. *Changes in glutamic acid distribution: effect of initial concentration of internal glutamic acid in* Staphylococcus aureus

(Cells grown for 16 hr. at 30° in deficient medium; made into washed suspension and divided into portions *A* and *B*; each portion incubated for 1 hr. at 37° in buffered salt solution, *A* with 1% glucose, *B* with 1% glucose and 10 μmol. sodium glutamate/ml. Organisms centrifuged down, washed once in distilled water and resuspended for treatment as in Table 3. Results expressed as change (μmol.) in glutamate/116 mg. dry wt. of cells.)

	Cells pretreated with glucose			Cells pretreated with glucose + glutamic acid	
Initial concentration of internal free glutamic acid (μmol./116 mg. dry wt. of cells)	13·2	13·2	13·2	54·5	54·5
Incubation mixture	Glucose + glutamate	Glucose + glutamate + amino-acid mixture	Glucose + amino-acid mixture	Glucose	Glucose + amino-acid mixture
Internal free glutamate of cells	+32·2	−5·8	−10·7	−17·0	−32·9
External free glutamate of medium	−56·6	−5·35	+ 3·8	+ 1·8	+13·0
Combined glutamate of cells	− 7·1	+6·35	+ 4·9	+ 2·05	+16·5
Balance	−28·4	−4·8	0·0	−13·8	− 0·4

Table 6. *Effect of concentration of internal free glutamic acid on rate of increase in combined glutamate in* Staphylococcus aureus

(Collected results on batches of cells pretreated with glucose or glucose + glutamic acid (as in Table 5) and then incubated with glucose and a mixture of eighteen amino-acids (without glutamic acid) as in Table 3. Decrease in internal free glutamate and increase in combined glutamate determined during 1 hr. at 37° and results expressed as change in μmol. glutamate/100 mg. dry wt. of cells.)

	Internal free glutamic acid		External glutamate concentration (μmol./ml.)	Increase in combined-glutamate (μmol./hr./100 mg. cells)
Exp.	Initial (μmol.)	Decrease (μmol.)		
a	11·3	9·2	0	5·1
	—	5·05	1·34	5·45
	47·0	28·4	0	14·3
b	13·2	11·0	0	3·4
	—	6·3	1·34	6·5
c	15·7	9·45	0	14·3
	—	5·37	1·34	12·1
	61·0	26·5	0	19·2
d	51·0	22·4	0	14·0

and glucose. Consequently, the difference between the free glutamic acid concentrations within the previously saturated and the control cells decreases rapidly on incubation. Fig. 2 shows that the rate of combination of glutamic acid in the cells initially saturated with free glutamic acid is higher than that of the control, but that the rate falls as the internal free glutamic acid concentration falls.

Table 6 shows collected results relating the rate of increase of cell-combined glutamate to the initial concentration of free glutamic acid within the cells and to the presence or absence of external glutamic acid. It is clear that a high internal concentration of free glutamic acid can be correlated with a rapid rate of formation of combined glutamate. The rate of combination is not affected to any marked extent by the presence of external glutamic acid at the concentration used (see below), but its presence decreases the rate of disappearance of free glutamic acid inside the cells. In some cases the increase in combined glutamate of the cells is approximately equal to the decrease in the amount of internal free glutamic acid, but in other cases it would appear that the combined glutamate can be drawn from either the internal or external medium.

In the absence of external sources of glutamic acid, the rate at which the internal concentration of free glutamic acid decreases during the formation of combined glutamate is frequently greater than the rate of appearance of combined glutamate in the cells. Fig. 3 shows that the rate of disappearance is greater when incubation takes place in the presence of glucose and an amino-acid mixture (free from glutamic acid) than in the presence of glucose or buffered salt alone. When *Staph. aureus* cells are incubated in buffered salt solution, a slow diffusion of internal glutamic acid occurs and 90% of the amino-acid disappearing from within the cell can be recovered from the external medium. If incubation takes place in the presence of glucose, the outward

diffusion is greatly decreased. This effect of glucose is the opposite of that which takes place with suspensions of *Strep. faecalis* (Gale, 1947*a*, 1948). The glutamic acid which disappears from *Staph. aureus* in the presence of glucose and amino-acids can be recovered to the extent of approximately 50 % from the external medium, while approximately 35 % is accounted for by increase in combined glutamate of the cells. It appears that glutamic acid passes out of the cell more easily under the conditions in which its combination occurs than when incubation takes place in a simple salt or salt and glucose medium.

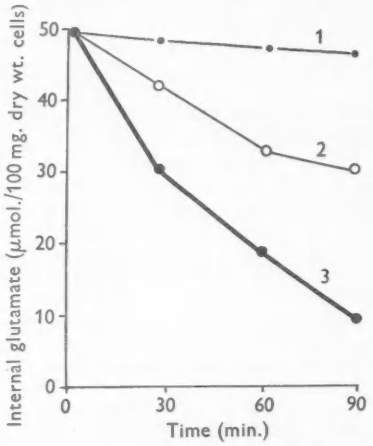

Fig. 3. Rate of decrease of internal free glutamate in *Staphylococcus aureus*. Cells incubated at 37° in (1) buffered salt solution containing 1 % glucose, (2) buffered salt solution alone, (3) buffered salt solution containing 1 % glucose and a mixture of 18 amino-acids (no glutamic acid) each at final concentration of 0·2 mg./ml. Samples taken at intervals, cooled rapidly and free glutamate content determined.

Effect of external concentration of free glutamic acid on rates of accumulation of free and combined glutamic acid in the cells

It has been shown above that the formation of combined glutamate in the cells is accompanied by cessation of the accumulation of free glutamic acid. In the experiments quoted the amount of glutamic acid present in the external medium has been of the same order as the amount of each of the other amino-acids added. It has also been shown that the internal concentration of free glutamic acid plays a significant part in the rate of formation of combined glutamate. The question arises whether high external concentrations of glutamic acid would restore accumulation of the free amino-acid and what effect this would have on the rate of formation of combined glutamate. Table 7 shows results obtained when *Staph. aureus* cells were incubated with a constant concentration of the amino-acid mixture but with the external concentration of glutamic acid increased 10–100 times the value previously used. When the external concentration is low, the internal concentration of free glutamic acid falls during the formation of combined glutamate; increasing the external concentration slows down the disappearance of internal free glutamic acid and, at very high values, restores a small rate of accumulation of the free amino-acid. An increase in the external glutamic acid concentration from 1·34 to 13·4 μmol./ml. results in a significant increase in the rate of formation of combined glutamate, but higher concentrations appear to be inhibitory and no increase in the combined glutamate of the cells occurs when the external concentration is 134 μmol./ml.

DISCUSSION

In an early paper of this series (Gale, 1947*b*) it was shown that the free glutamic acid content of *Staph. aureus* cells in growing cultures increased as growth progressed and reached a maximum when growth

Table 7. *Effect of external glutamic acid concentration on rates of accumulation of free glutamic acid and formation of combined glutamate in* Staphylococcus aureus

(Conditions as for Table 3. Final concentration of each component of amino-acid mixture = 0·16 mg./ml. Control = glucose, sodium glutamate, buffered saline solution.)

External concentration sodium glutamate (μmol./ml.)	Rate of free glutamate accumulation (μmol./100 mg. dry wt. cells/hr.)		Rate of increase in combined glutamate (μmol./100 mg. dry wt. cells/hr.)	
	Control	Amino-acids present	Control	Amino-acids present
1·34	+39·5	−6·75	0	7·7
13·4	+43·7	+0·45	0	8·3
45·0	—	+6·4	0	6·6
1·34	+28·0	−6·7	0	5·4
13·4	—	−0·7	0	6·15
45·0	—	+2·5	0	0·93
134·0	+43·7	+4·0	0	0·0

ceased. It was also shown that the rate of accumulation of free glutamic acid within the harvested cells, tested in the absence of other amino-acids, was constant and independent of the growth phase. Since the steady state concentration of free glutamic acid within the cells is determined by a number of processes (Gale & Mitchell, 1947) and there is a reciprocal relation between the free and combined glutamic acid contents of the cells, it was deduced that the free glutamic acid accumulated within the cells before condensation into protein, and that a high rate of protein synthesis in 'young' growing cells lowered the concentration of free glutamic acid inside these cells. From the facts put forward in this communication it is clear that the relationship between accumulation of free glutamic acid and formation of combined glutamate in the cell cannot be as simple as previously suggested.

If washed cells are incubated in the presence of glucose and a mixture of amino-acids, glutamic acid enters into a combined form within the cell from which it can be released by acid hydrolysis. When these results are considered in conjunction with those of Hotchkiss (1947), it seems reasonable to suppose that the combined form is of a protein or protein + peptide nature. If glutamic acid is the only amino-acid present, it becomes concentrated in the free state inside the cell and no peptide synthesis occurs. If, during the accumulation of the free glutamic acid inside the cell, an amino-acid mixture, each component of which is in approximately the same concentration as the external glutamic acid, is added, the accumulation appears to cease and combined glutamate is formed. If, however, the external concentration of glutamic acid is greatly in excess of the concentration of the components of the amino-acid mixture, then some accumulation of free glutamic acid occurs within the cell, but the formation of combined glutamate is suppressed. There appears, therefore, to be competition between the processes involved in the accumulation of the free amino-acid and its combination into cell material, the former process taking place when glutamic acid is the major component of the external medium, the latter when a complete and balanced amino-acid mixture forms the external medium.

Comparison of the changes in glutamic acid distribution that take place in the two cases shows that, when the formation of combined glutamate (protein synthesis?) is occurring, the concentration of internal free glutamic acid decreases instead of increases, and this is accompanied by a marked decrease in the rate of withdrawal of glutamic acid from the external medium. The concentration of free glutamic acid within the cells is determined by the balance between the rate at which it enters the cell and the rate at which it is metabolized within the cell; the onset of synthesis would therefore cause a decrease in the rate of internal accumulation, but this should not be accompanied by a fall in the rate at which the amino-acid enters the cell. Two explanations appear possible: either the presence of other amino-acids in the external medium must decrease the rate of entry of glutamic acid into the cell or, alternatively, the passage of glutamic acid into the cell involves a metabolic stage which is also part of the process of peptide bond formation so that glutamic acid does not accumulate in the free state within the cell when the presence of other amino-acids renders peptide bond formation possible.

SUMMARY

1. When *Staphylococcus aureus*, in washed suspensions, is incubated in the presence of glucose and glutamic acid, free glutamic acid accumulates within the cells. A certain amount of the amino-acid is metabolized but no formation of combined glutamate occurs. In the presence of a complete and balanced mixture of amino-acids, the accumulation of free glutamic acid within the cells decreases and may cease, the amount of glutamic acid withdrawn from the medium in a given time is markedly less than that withdrawn when glutamic acid is the only amino-acid present, and an increase occurs in the amount of combined glutamate in the cells.

2. The rate at which the combined glutamate of the cells increases can be correlated with the initial concentration of free glutamic acid within the cells.

3. If the external glutamic acid concentration is raised to approximately 100 times that of the other components of the amino-acid mixture, some accumulation of free glutamic acid occurs, but the formation of cellular combined glutamate is suppressed.

4. Whether glutamic acid accumulates in the free state within the cell or enters into combination within the cell depends upon the balance between glutamic acid and the other amino-acids in the medium.

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The Assimilation of Amino-acids by Bacteria

12. THE ACTION OF INHIBITORS AND ANTIBIOTICS ON THE ACCUMULATION OF FREE GLUTAMIC ACID AND THE FORMATION OF COMBINED GLUTAMATE IN *STAPHYLOCOCCUS AUREUS*

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The passage of glutamic acid into *Staphylococcus aureus* takes place only when energy is supplied by some exergonic metabolism such as fermentation or respiration (Gale, 1947). It is possible to prevent the passage of glutamic acid into the cell without affecting fermentation by the addition of inhibitors such as sodium azide, 2:4-dinitrophenol and 8-hydroxyquinoline (Gale, 1949, 1951*a*). Penicillin has no action on the passage when this takes place in washed cells, but if added in bactericidal concentrations to the growth medium 60–90 min. before harvesting, it causes a complete inhibition of the entry of glutamic acid into the harvested cells, although their rate of fermentation is normal (Gale & Taylor, 1947). When washed cells of *Staph. aureus* are incubated with a mixture of amino-acids and glucose they synthesize cell protein (Hotchkiss, 1947), but the accumulation of free glutamic acid is greatly decreased (Gale, 1951*b*). The present communication deals with the action of a number of inhibitors and antibiotics on the processes of accumulation of free glutamic acid on the one hand, and of the increase in combined glutamate of the cells occurring when incubation takes place in the presence of a balanced mixture of amino-acids on the other.

METHODS

The methods used in these investigations were the same as those previously described (Gale, 1947, 1951*b*). The organism used throughout was *Staph. aureus* Duncan (Gale & Taylor, 1947). The authors are indebted to the Lederle laboratories for a sample of crystalline aureomycin, to Dr L. A. Sweetman for synthetic chloramphenicol (chloromycetin) and to Merck and Co. for streptomycin. Penicillin solutions were made from a commercial sample of crystalline benzylpenicillin assayed by the makers at 1650 Oxford units/mg.

RESULTS

Inhibitors effective on the processes in washed suspensions. Table 1 shows that sodium azide and 2:4-dinitrophenol affect the accumulation of free glutamic acid and the rate of increase in combined glutamate of the cells to significantly the same extent. 8-Hydroxyquinoline, which appears to inhibit the accumulation of free glutamic acid by combining with and inactivating an essential metal replaceable by manganese (Gale, 1949), is a more effective inhibitor of the accumulation of the free amino-acid than of its combination within the cell.

Aureomycin and chloramphenicol are alike in that they inhibit the formation of combined glutamate more effectively than the accumulation of glutamic acid in the free state. Loomis (1950) has shown that aureomycin, in concentrations greater than 100 μg./ml. (0·2 mM), will uncouple phosphorylation in mitochondrial preparations and should be grouped with azide and dinitrophenol in this respect. It is interesting to note that the accumulation of free glutamic acid is also inhibited by aureomycin at this concentration, although the growth of *Staph. aureus* is sensitive to much smaller concentrations. Chloramphenicol produces 10 % inhibition of free glutamic acid accumulation at a concentration of 1·5 mM (500 μg./ml.), and Loomis (1950) states that this antibiotic is not effective in uncoupling phosphorylation in his experiments. Chloramphenicol inhibits growth of *Staph. aureus* at a concentration of 1–5 μg./ml., and these concentrations cause 77–96 % inhibition of the formation of combined glutamate.

Streptomycin is a weak inhibitor of both processes, being ineffective against either at concentrations less than 0·1 mM, whereas growth is inhibited at a concentration of 0·001 mM. In the higher range of concentrations, streptomycin is a more effective inhibitor of combined glutamate formation than of the accumulation of the free amino-acid. Sulphathiazole in saturated solution had no significant inhibitory action on either process.

In all cases tested, the inhibitors were effective against glutamic acid metabolism at concentrations ineffective against glucose fermentation.

Action of penicillin

Penicillin has no action on the accumulation of free glutamic acid in washed suspensions of *Staph. aureus*. If 10 units penicillin/ml. are added to the growth medium after 4 hr. growth at 37° and the

Table 1. *Inhibition of free glutamic acid accumulation and of the formation of combined glutamate in* Staphylococcus aureus

(*Growth:* growth (++) or inhibition (−) when *Staph. aureus* Duncan inoculated into casein-digest broth containing 1% glucose, 0·1% marmite and inhibitor as shown; size of inoculum = approx. 10^8 cells/5 ml. medium.

Glucose fermentation: tested in Warburg manometers containing washed cells of dry wt. = approx. 3 mg., 0·03 M-$NaHCO_3$, 0·05 M-glucose and water or inhibitor to 3·0 ml.; filled with N_2 gas containing 5% CO_2.

Glutamate accumulation: organisms harvested from deficient medium and incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml.; increase in free internal glutamic acid determined and expressed as μmol. glutamic acid/100 mg. dry wt. of cells/hr.

Increase in combined glutamate: experiment carried out in parallel with accumulation but with addition of mixture of eighteen amino-acids each at a final concentration of 0·2 mg./ml. Free and total glutamic acid of cells determined before and after incubation; total glutamic acid determined after 18 hr. hydrolysis in boiling 5 N-HCl; results expressed as μmol. increase in combined glutamate/100 mg. dry wt. of cells/hr.)

Inhibitor	Concn. (mM)	Growth	Inhibition of glucose fermentation (%)	Glutamate accumulation (μmol./100 mg. dry wt. cells/hr.)	Glutamate accumulation Inhibition (%)	Increase in combined glutamate (μmol./100 mg. dry wt. cells/hr.)	Increase in combined glutamate Inhibition (%)
Sodium azide	0	++	.	27·0	.	6·7	.
	10·0	−	2	0	100	0	100
	3·3	++	0	16·2	43	1·56	65
2:4-Dinitrophenol	0	++	.	30·5	.	8·17	.
	1·0	−	0	3·8	87	1·66	80
	0·1	++	0	22·1	25	6·0	26
8-Hydroxyquinoline	0	++	.	39·5	.	6·98	.
	0·3	−	20	14·8	62	6·62	5
	0·03	++	0	28·6	27	6·8	2·5
Aureomycin	0	++	.	30·1	.	6·16	.
	0·2	−	19	0	100	0	100
	0·07	−	2	21·6	29	0	100
	0·014	−	0	29·2	3	3·08	50
Chloramphenicol (Chloromycetin)	0	++	.	43·6	.	7·64	.
	1·5	−	0	39·0	10	0	100
	0·15	−	0	43·4	0	0	100
	0·015	+	0	.	.	0·3	96
	0·003	++	0	.	.	1·84	77
	0·001	++	0	.	.	6·03	21
Streptomycin	0	++	.	33·0	.	9·15	.
	1·0	−	0	31·7	4	5·04	45
	0·1	−	0	33·0	0	6·72	26
	0·01	−	0	.	.	8·6	5
	0·001	++	0	.	.	9·4	0
Sulphathiazole	0	++	.	30·5	.	6·16	.
	Satd.	+	0	30·5	0	6·15	0

cells harvested after 90 min. further incubation, it is found that the washed cells can no longer accumulate free glutamic acid (Gale & Taylor, 1947). Hotchkiss (1950) states that washed *Staph. aureus*, aerated for 1 hr. and then incubated with glucose and casein hydrolysate, synthesizes cellular protein but that, if 50 units penicillin/mg. dry weight of cells are added, the removal of amino-acids from the medium continues but no increase in cell protein occurs, peptides accumulating in the medium instead. Washed suspensions of *Staph. aureus* Duncan, grown in deficient medium, were incubated with and without penicillin in the presence of glucose and a mixture of nineteen amino-acids including glutamic acid under conditions previously described (Gale, 1951*b*).

Table 2 shows that the presence of penicillin has no effect on the rate of increase of combined glutamate of the cells and no accumulation of glutamyl peptides could be demonstrated in the external medium. If, however, penicillin is added to the growth medium and the cells harvested within 30–60 min. after the addition (before the passage of glutamic acid into the cell has been completely inhibited), it is found that the cells can no longer form combined glutamate (Table 3). Analysis of the changes in glutamic acid distribution during the incubation shows, however, that free glutamic acid has disappeared from the medium and that a proportion, which varies between 20 and 45%, can be recovered by acid hydrolysis of the supernatant

Table 2. *Effect of penicillin on distribution of glutamic acid during incubation of* Staphylococcus aureus *with glucose and amino-acid mixtures*

(*Staph. aureus* Duncan grown for 16 hr. at 30° in deficient medium, washed once in water and made into suspension in water, dry wt. of cells = 21·2 mg./ml. 5·0 ml. suspension taken for each experiment and incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, 1·34 μmol. sodium glutamate/ml., mixture of eighteen amino-acids each at final concentration 0·2 mg./ml. (Gale, 1951*b*) with and without 100 units penicillin/ml. as below. Final suspension density = 2·12 mg. dry wt. of cells/ml. Free and combined glutamate of cells and supernatant determined before and after incubation; changes expressed as μmol. glutamate/106 mg. dry wt. of cells.)

Incubation mixture	Glucose	Glucose + glutamate	Glucose + glutamate + penicillin	Glucose + glutamate + amino-acid mixture	Glucose + glutamate + amino-acid mixture + penicillin
Internal free glutamate of cells	+0·2	+29·8	+27·5	−6·91	−7·82
External free glutamate of medium	+1·48	−48·1	−42·7	−7·4	−2·8
Combined glutamate of cells	−0·54	− 1·5	−13·8	+8·8	+6·6
Combined glutamate of medium	+1·1	+ 2·7	+ 1·3	+2·0	0·0
Balance	+2·37	−17·2	−27·6	−3·44	−4·15

Conditions as above with samples removed for estimation at times indicated.

Time (min.)	Increase in combined glutamate of cells (μmol./79 mg. dry wt. cells)		Glutamic acid balance expressed as % initial total	
	Normal	Penicillin present	Normal	Penicillin present
Initial	(27·2)	(27·2)	—	—
60	5·6	7·2	99·0	103·1
90	11·1	11·7	95·8	99·2
120	15·4	13·9	94·3	85·0
150	18·8	18·8	88·7	88·3

Table 3. *Glutamic acid metabolism of penicillin-treated* Staphylococcus aureus

(Cells grown in deficient medium at 37° for 4 hr.; 10 units penicillin/ml. medium added and cells harvested 1 hr. after addition. Washed suspensions made and treated as in Table 2. Changes in glutamate distribution during 1 hr. at 37° expressed as μmol. glutamate/100 mg. dry wt. of cells.)

Incubation mixture	Glucose	Glucose + glutamate	Glucose + glutamate + amino-acid mixture
Internal free glutamate of cells	− 1·57	+ 0·9	−2·01
External free glutamate of medium	+ 1·03	−41·5	−1·6
Combined glutamate of cells	−13·8	− 8·6	−6·3
Combined glutamate of medium	—	+19·4	0
Balance	—	−29·8	−9·9

medium. It seems probable that conditions have arisen in this case which have results similar to those reported by Hotchkiss (1950).

SUMMARY

1. The action of inhibitors has been studied on the growth, glucose fermentation, accumulation of free glutamic acid, and formation of cellular combined glutamate in *Staphylococcus aureus*. In all cases quoted, the inhibitors suppressed the accumulation of free glutamic acid or its combination in the cell at concentrations ineffective against fermentation.

2. Of the two processes (*a*) accumulation of free glutamic acid, (*b*) formation of cellular combined glutamate, sodium azide and 2:4-dinitrophenol are equally effective against both; 8-hydroxyquinoline is more effective against (*a*) than (*b*); aureomycin and chloramphenicol are markedly more effective as inhibitors of (*b*) than (*a*); streptomycin affects (*b*) at concentrations markedly higher than those required to prevent growth.

3. There is a close correlation between the inhibitory action of chloramphenicol on (*b*) and its action as growth inhibitor.

4. Penicillin has no action on the processes in washed suspensions; addition of penicillin to the medium 30–60 min. before harvesting results in complete inhibition of (*a*) and (*b*).

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The Differential Determination of Mixtures of *p*-Aminosalicylic Acid and Sulphetrone or Sulphonamides in Body Fluids

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Since the introduction of *p*-aminosalicylic acid (PAS) to the chemotherapy of tuberculosis, several methods for its estimation have been described. These methods have depended on the presence of the free aryl amino group in the molecule, and have the disadvantage of a lack of specificity since free aryl amino groups are of frequent occurrence in synthetic drugs. Difficulties may therefore arise in estimating one in the presence of another. This can occur for instance in the combined use of PAS and diaminodiphenylsulphone derivatives, such as sulphetrone, (tetrasodium 4:4′-bis(γ-phenyl-*n*-propylamino)diphenylsulphone-αγα′γ′-tetrasulphonate), or PAS, and sulphonamide derivatives. All the drugs may be estimated singly by the standard method described by Bratton & Marshall (1939) of diazotizing and coupling with *N*-1-naphthylethylenediamine, and an examination of the visible absorption spectra of the coloured solutions (Fig. 1) shows that no differentiation is possible for PAS, sulphetrone, sulphanilamide and sulphathiazole, since the peak absorptions all lie in the same spectral area. This was also found to be true of other sulphonamides examined, namely, sulphadiazine, sulphaguanidine and sulphamezathine. To enable differentiation to be made, alternative methods of estimating the PAS alone have been examined. These have included the use of the reaction with ferric iron, coupling with *p*-dimethylaminobenzaldehyde, and coupling with diazonium salts of various amines in alkaline solution.

Reaction with ferric iron. PAS gives the characteristic salicylate reaction in neutral or faintly acid solution, producing a coloured complex with solutions of ferric salts. Sulphetrone and the sulphonamides do not give this reaction. It has been used for the estimation of PAS in urine (Venkatamaran, Venkatamaran & Lewis, 1948) and may be adapted to the estimation of high concentrations in blood filtrates. Unfortunately the colour produced is not intense, making the method somewhat insensitive and of little value for general purposes.

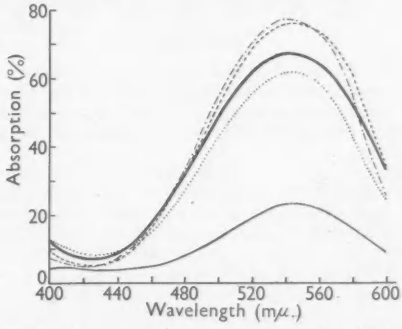

Fig. 1. Visible absorption spectra after diazotizing and coupling with *N*-1-naphthylethylenediamine. Sulphetrone and PAS (1·0 mg./100 ml. of each), ——; PAS (1·0 mg./100 ml.), ——; sulphathiazole (0·2 mg./100 ml.), - - - - -; sulphanilamide (0·2 mg./100 ml.), – · – · –; sulphetrone (1·0 mg./100 ml.), ...

Coupling with p-*dimethylaminobenzaldehyde.* *p*-Dimethylaminobenzaldehyde reacts with free aryl amino groups yielding characteristic lemon-yellow compounds. This has been applied by Venkatamaran *et al.* (1948) and others to the estimation of PAS. The sulphonamides give a similar reaction, and comparison of the visible absorption spectra of the coloured solutions at equivalent concentration shows the intensities of the colours to be approximately equal (Fig. 2). Sulphetrone also gives a colour with *p*-dimethylaminobenzaldehyde, but it is much less intense, and concentrations in the coloured solution of less than 0·2 mg./ml. do not differ significantly from the blank. At the dilutions

necessary for the estimation of PAS (generally 1/100), this would correspond to concentrations of sulphetrone in the blood (at least 20 mg./100 ml.) much higher than those normally encountered. Thus this method is satisfactory for the estimation of PAS in the presence of sulphetrone (Short, 1949), but not in the presence of sulphonamides.

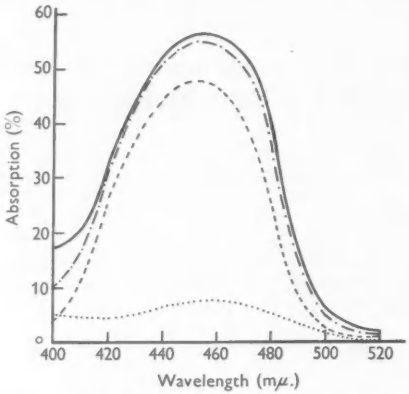

Fig. 2. Visible absorption spectra after coupling with *p*-dimethylaminobenzaldehyde. PAS (0·2 mg./100 ml.), ——; sulphathiazole (0·2 mg./100 ml.), - - - - -; sulphanilamide (0·2 mg./100 ml.), −·−·−; sulphetrone (0·5 mg./100 ml.), ...

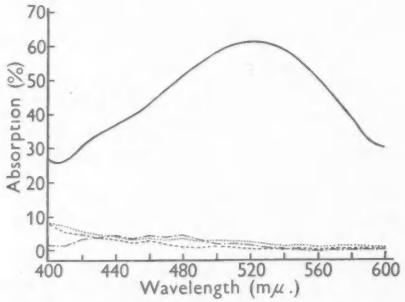

Fig. 3. Visible absorption spectra after coupling with diazo-*p*-nitraniline. PAS (0·8 mg./100 ml.), ——; sulphathiazole (16·0 mg./100 ml.), - - - - -; sulphanilamide (16·0 mg./100 ml.), −·−·−; sulphaguanidine (16·0 mg./100 ml.), ...

Coupling with diazo solutions. PAS couples with solutions of the diazonium salts of various amines in alkaline solution to give coloured compounds of various intensities. Diazotized sulphanilic acid added to a solution of PAS followed by sodium hydroxide gives an orange, and diazotized *p*-nitraniline similarly gives a deep-red colour. Fig. 3 compares the visible absorption spectra for three sulphonamides and PAS after coupling with diazotized *p*-nitraniline and making alkaline with sodium hydroxide. Solutions of 16 mg./100 ml. of the sulphonamides show absorptions which do not differ significantly from that of the blank solution. This method is suitable for general purposes and gives a valuable means of determining PAS, in the presence of sulphonamides, although its sensitivity is only two-fifths of that employing *p*-dimethylaminobenzaldehyde.

EXPERIMENTAL

Estimation of PAS in blood, urine and tissue filtrates by coupling with p*-dimethylaminobenzaldehyde*

The extension of the *p*-dimethylaminobenzaldehyde method used by Venkatamaran *et al.* (1948) to estimate PAS in blood and tissue filtrates has been studied in detail. The reaction is very sensitive to changes in pH and for maximum colour development the pH is critical, and should be between 2·0 and 2·2 (Table 1).

Table 1. *Effect of* pH *on the colour intensity of the product of PAS and* p*-dimethylaminobenzaldehyde*

(PAS 0·1 mg./100 ml.; Ilford filter no. 601.)

pH	Absorption (%)
3·72	18·5
3·35	22·9
3·00	25·5
2·50	29·5
2·15	31·9
2·06	32·2
1·87	30·5
1·55	26·8
1·42	20·2

A suitable filtrate is obtained by diluting with water and trichloroacetic acid so that the final concentration of the acid is 4·0% (w/v).

Reagents

Trichloroacetic acid. 12% (w/v) Aqueous solution.

p-*Dimethylaminobenzaldehyde solution. p*-Dimethylaminobenzaldehyde (2·0 g.) dissolved in 100 ml. glacial acetic acid is added to 100 ml. 4 M-sodium acetate. When 0·5 ml. of this reagent is added to 3·0 ml. water and 1·5 ml. trichloroacetic acid solution, the pH should lie between 2·0 and 2·2. If necessary, adjustment is made with either the *p*-dimethylaminobenzaldehyde solution in glacial acetic acid or 4 M-sodium acetate.

Standard PAS solution. PAS is stable in solution only as the Na salt. The acid is purified by twice recrystallizing from ethanol and drying over P_2O_5. A standard solution is prepared by suspending 100 mg. of the pure acid in a little water, warming gently with 4 drops of 20% (w/v) Na_2CO_3 solution and adjusting the volume to 100 ml. The standard solution contains 1 mg. PAS/ml.

Production of colour

Blood (0·1 ml.) is mixed with 5·9 ml. distilled water, 3·0 ml. of trichloroacetic acid is added, and after thorough mixing, the whole is filtered through a Whatman no. 5 filter

Table 2. *Recovery of PAS added to blood, estimated by coupling with* p-*dimethylaminobenzaldehyde*

(All results expressed as percentage recoveries are given, in this and subsequent tables, as the mean of six estimates ±S.E., with the range underneath in parentheses.)

PAS added (mg./100 ml.)	PAS recovered (%)		
	PAS alone	PAS with sulphetrone (5 mg./100 ml.)	PAS with sulphetrone (10 mg./100 ml.)
1·0	100·2±0·7 (98·0–102·0)	100·4±0·8 (98·0–104·0)	100·8±2·5 (90·5–105·0)
5·0	98·3±1·4 (96·0–102·0)	98·4±1·3 (95·0–104·0)	102·4±1·1 (99·6–106·0)
10·0	100·7±0·8 (98·0–104·0)	100·7±0·7 (98·0–103·5)	102·7±1·5 (99·0–108·0)
20·0	100·5±1·9 (96·0–107·0)	98·1±1·1 (94·0–100·5)	99·6±0·7 (98·0–102·5)

Table 3. *Recovery of PAS added to urine estimated by the* p-*dimethylaminobenzaldehyde method*

PAS added (mg./100 ml.)	PAS recovered (%)		
	PAS alone	PAS with sulphetrone (50 mg./100 ml.)	PAS with sulphetrone (100 mg./100 ml.)
10·0	97·5±1·5 (90·0–102·0)	103·9±2·8 (99·0–115·0)	101·8±1·4 (98·0–107·0)
50·0	100·2±0·7 (98·0–102·0)	100·3±1·3 (98·0–106·0)	96·0±1·8 (94·0–102·0)
100·0	100·3±1·6 (94·0–104·0)	98·3±1·1 (94·0–102·0)	104·4±1·6 (100·0–108·0)
200·0	102·1±1·5 (96·5–106·5)	95·8±1·2 (92·0–98·5)	97·5±1·2 (92·0–100·0)

paper. *p*-Dimethylaminobenzaldehyde reagent (0·5 ml.) is added to 4·5 ml. of the filtrate and the colour measured absorptiometrically, using an Ilford filter no. 601. (Maximum absorption is at 454 mμ.; see Fig. 2.) The colour may be measured 10 min. after the addition of the reagent and is stable for at least 3 hr.

The dilution of blood is 1/100. For PAS concentrations below 2·0 mg./100 ml., a dilution of 1/50 is better and for concentrations above 20 mg./100 ml. greater dilution is necessary, the volume of blood and distilled water being adjusted in each case so that the final concentration of trichloroacetic acid remains the same. PAS is bound to plasma proteins to a considerable extent. Way, Smith, Howie, Weiss & Swanson (1948) have shown that at plasma levels from 4 to 10 mg./100 ml. blood, approximately 50–60% is bound to plasma proteins. It is important that the original dilution of the blood is sufficient to free this combined portion of the drug. The dilution described (a minimum of 1/50) is ample for this purpose. With dilutions lower than 1/20 some of the drug is lost on the protein precipitate and recoveries are lower.

To estimate the drug in urine, preliminary dilution in distilled water is necessary. The diluted urine is then treated in the same way, trichloroacetic acid being added so that the final concentration is 4·0% (w/v).

Standard curves covering the final concentration range 0–0·2 mg./100 ml. in suitably diluted blood or urine are prepared from the standard solution in an exactly similar manner.

The concentration of PAS in tissue may be estimated by grinding 2 g. of the sample with washed silver sand in a glass mortar, mixing with 10 ml. of distilled water and leaving to macerate for 1 hr. The contents of the mortar are then washed into a stoppered cylinder, the volume finally being adjusted to 60 ml. with distilled water. Trichloroacetic acid solution (30 ml.) is then added, the cylinder stoppered and well shaken. The mixture is filtered through a Whatman no. 5 filter paper and 4·5 ml. of the filtrate coloured in the usual way. The dilution of the tissue is 1/50. A further dilution of the filtrate with 4% trichloroacetic acid solution may be made if necessary.

Table 4. *Extent of darkening of* p-*dimethylaminobenzaldehyde reagent*

(The blank was prepared from 3·0 ml. distilled water, 1·5 ml. trichloroacetic acid and 0·5 ml. reagent. Ilford filter no. 601.)

Time after preparation of reagent (weeks)	Transmission of blank with reagent (%)		
	A	*B*	*C*
0	89·5	92·1	90·7
1·5	—	—	90·7
3	—	—	90·3
8	—	—	88·6
19	—	87·1	—
32	62·1	—	—

Tables 2 and 3 show the recoveries of PAS from blood and urine. Recoveries are expressed as percentages, and the values given are the mean of six determinations in each case.

PAS is equally well estimated at all concentrations used, the overall mean recovery being 99·9% in blood and 100% in urine.

A solution of *p*-dimethylaminobenzaldehyde darkens on keeping, but this effect is minimized by preparing the solution in acetate buffer as described. Table 4 shows the extent of darkening of various solutions over a period of 6 weeks. The reagent is suitable for absorptiometric work for at least 4 weeks and for direct visual comparison for longer periods if a similarly prepared blank is used.

Determination of PAS-sulphetrone mixtures

Sulphetrone is usually determined by diazotizing and coupling with *N*-1-naphthylethylenediamine (Brownlee, Green & Woodbine, 1948). PAS produces a similar colour, and the method has been applied to its estimation (Way *et al.* 1948; Newhouse & Klyne, 1949).

The reaction with PAS is less sensitive than that with sulphetrone (Fig. 1), but the colour produced by concentrations of the drug normally encountered in body fluids is sufficient to interfere with the estimation of sulphetrone. A differential method for estimating drugs by using the *p*-dimethylaminobenzaldehyde and naphthylethylenediamine reactions has been studied.

Sulphetrone gives only a faint colour with *p*-dimethylaminobenzaldehyde (Fig. 2), and at the dilutions generally employed PAS may be estimated accurately in the presence of the sulphetrone. Tables 2 and 3 give the results of estimation of PAS added to blood and urine containing 5 and 10 mg./100 ml. of sulphetrone, and 50 and 100 mg./100 ml. PAS respectively. Recoveries, expressed as percentages, are the mean of six determinations in each case. PAS is equally well estimated at all concentrations and in all the mixtures employed, overall recoveries being within $100 \pm 0{\cdot}25\%$ in blood and in urine.

The 'total diazotizable substances' (sulphetrone and PAS) are next determined by the method of Brownlee *et al.* (1948). Standard curves for sulphetrone of final concentration range 0–1 mg./100 ml. (Fig. 4, curve *A*) and PAS of final concentration range 0–2 mg./100 ml. (Fig. 4, curve *B*) are prepared similarly from standard solutions in corresponding dilutions of blood and urine.

Calculation of results. The 'total diazotizable concentration' in terms of sulphetrone is determined by reading the percentage absorption from curve *A*. The absorption given by the concentration of PAS in the filtrate (independently determined by the *p*-dimethylaminobenzaldehyde method) is read from curve *B*. The concentration of sulphetrone equivalent to this absorption is then determined from curve *A*. This gives the correction for the colour due to the PAS and subtraction of this from the 'total diazotizable concentration' gives the actual concentration of sulphetrone in the filtrate. Typical standard curves *A* and *B* and a specimen protocol of the results are given in Fig. 4.

Recoveries obtained by this method when sulphetrone was added to blood and urine containing varying concentrations of PAS, are given in Tables 5 and 6.

Satisfactory recoveries from urine were obtained in all the mixtures employed, the overall mean recovery being 100·1%. The results with blood show a greater variation, the mean percentage recovery with the lower concentration of sulphetrone (5 mg./100 ml.) of 100·0% indicates complete recovery of the drug. The corresponding figure for the higher sulphetrone concentration (10 mg./100 ml.) is 107·1%; although the difference between this figure and 100 is statistically significant, it is not sufficiently great to be of clinical importance, especially as such high blood concentrations are to be avoided in clinical practice. It may be that the high sulphetrone concentration prevents complete removal of the PAS from the plasma protein, resulting in its low estimate. Support is lent to this hypothesis by the fact that mixtures of higher concentrations in urine do not give these high recoveries.

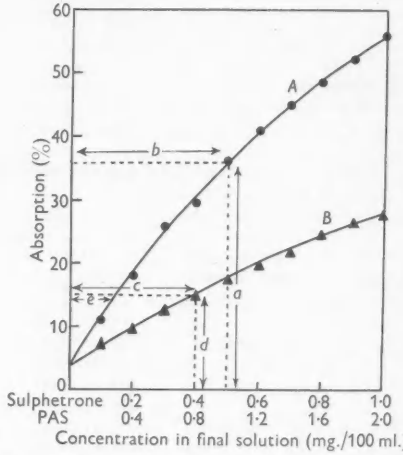

Fig. 4. Standard curves for the estimation of sulphetrone (curve *A*) and PAS (curve *B*) by diazotizing and coupling with *N*-1-naphthylethylenediamine. Ilford filter no. 605. An absorption of 35·9% (*a*) corresponds to a 'total diazotizable concentration' in terms of sulphetrone of 0·5 mg./100 ml. (*b*) see curve *A*. Let the concentration of PAS in the final filtrate, calculated after independent determination of the concentration of PAS in the sample by coupling with *p*-dimethylaminobenzaldehyde, be 0·8 mg./100 ml. (*c*). From curve *B*, the absorption corresponding to 0·8 mg./100 ml. PAS is 15·2% (*d*), and this percentage absorption corresponds to 0·15 mg. sulphetrone/100 ml. and the actual concentration of sulphetrone in the filtrate is given by subtraction of this value from the 'total diazotizable concentration' (0·5 − 0·15 = 0·35 mg. sulphetrone/100 ml.). Correction for the dilution then gives the concentration of sulphetrone in the sample.

*Estimation of PAS by coupling with diazotized-*p*-nitraniline*

Various methods of estimation depending on the coupling of PAS with diazo solutions have been described. The use of diazotized *p*-nitraniline is described by Tennent & Leland (1949), who employed a differential method of coupling in the presence of pyridine on the one hand and extracting the dye with pyridine on the other to eliminate interfering colours, and Street (1949), who diazotized both the PAS and *p*-nitraniline and then made alkaline with NaOH.

In attempts to apply these methods to mixtures containing PAS and sulphonamides, it was found that the colour pro-

Table 5. *Recoveries of sulphetrone added to blood in the presence of PAS*

Sulphetrone added (mg./100 ml.)	PAS concentration (mg./100 ml.) 1·0	5·0	10·0	20·0
	Sulphetrone recovered (%)			
5·0	99·2±1·7 (94·0–103·6)	98·8±1·9 (90·0–103·0)	103·0±1·0 (101·0–107·0)	99·0±1·9 (94·0–104·0)
10·0	105·5±0·8 (102·0–107·0)	108·5±1·9 (103·0–112·0)	106·8±1·3 (104·0–112·0)	107·5±2·2 (103·0–115·0)

Table 6. *Recoveries of sulphetrone added to urine in the presence of PAS*

Sulphetrone added (mg./100 ml.)	PAS concentration (mg/100 ml.) 1·0	5·0	10·0	20·0	200·0
	Sulphetrone recovered (%)				
5	94·9±0·7 (93·2–98·0)	100·6±1·0 (98·0–103·8)	97·8±1·6 (90·0–100·0)	103·3±3·5 (90·0–112·0)	—
10	96·5±0·8 (95·2–99·7)	95·2±0·8* (93·3–101·2)	101·6±1·8 (94·8–108·2)	106·5±3·5 (104·0–120·0)	—
100	—	—	—	—	96·8±1·2* (94·0–100·0)
150	—	—	—	—	105·6±1·8† (101·0–110·0)
200	—	—	—	—	101·8±0·9 (98·5–105·0)

* Four estimates. † Five estimates.

Table 7. *Recoveries of PAS in the presence of various sulphonamides*

(Concentration of sulphonamide 100 mg./100 ml. PAS estimated by the diazotized *p*-nitraniline method.)

PAS added (mg./100 ml.)	PAS recovered (%) PAS with sulphanilamide	PAS with sulphadiazine	PAS with sulphaguanidine
20·0	101·3±2·9 (91·5–106·0)	102·7±1·5 (99·0–107·0)	102·2±2·2 (95·0–110·5)
50·0	99·2±1·7 (91·2–104·0)	95·4±1·3 (90·4–100·0)	98·8±2·5 (92·0–108·0)
100·0	96·9±2·7 (92·5–110·0)	96·3±2·7 (90·0–103·5)	96·1±0·9 (94·0–100·0)

duced by the latter was due to excess nitrite added with the diazotized *p*-nitraniline solution. This diazotized the sulphonamide which then coupled with the reagent to give a coloured complex. If, however, the excess nitrite was removed from the diazotized *p*-nitraniline solution, no significant colour was produced in solutions containing at least twenty times the concentration of PAS suitable for measurement (Fig. 3).

Reagents

Trichloroacetic acid. 12% (w/v) Aqueous solution.

Sodium hydroxide. 2N.

Sodium nitrite. 0·3% (w/v) Aqueous solution. This should be freshly prepared each week.

Ammonium sulphamate. 1·5% (w/v) Aqueous solution.

p-*Nitraniline.* 0·05% (w/v) Solution in 0·1N-HCl.

Diazotized p-*nitraniline solution.* This should be freshly prepared by adding to 10·0 ml. *p*-nitraniline solution 1 ml. $NaNO_2$ solution, allowing to stand for 3 min. after thorough mixing, and then adding 0·3 ml. ammonium sulphamate solution. The reagent should be left for at least 5 min. after the addition of the sulphamate before use. It is stable for about 3 hr.

Production of colour

Blood (0·4 ml.) is added to 3·6 ml. distilled water and 3·0 ml. of trichloroacetic acid added. After thorough mixing, the protein precipitate is removed by filtering through a Whatman no. 5 filter paper. Diazotized *p*-nitraniline solution (0·5 ml.) is added to 3·5 ml. of the filtrate, the mixture allowed to stand for 3 min., and 1·0 ml. of NaOH solution then added. A deep-red colour develops immediately and, after 15 min., it is measured absorptiometrically, using an Ilford filter no. 604. (Maximum absorption is at 524 mμ.; see Fig. 3.)

The dilution of the blood is 1/25. For concentrations above 15 mg./100 ml. greater dilution is necessary, the volume of distilled water and blood being adjusted so that the ratio of

trichloroacetic acid, diazotized *p*-nitraniline and NaOH solutions remains the same.

To estimate the drug in urine, preliminary dilution in distilled water may be necessary. The diluted urine is then treated in the same way, the ratio of the reagents being kept as above.

Corresponding standard curves in blood and urine of final concentration range 0–1·0 mg./100 ml. are prepared similarly, using dilutions of the standard solution.

The recoveries of PAS estimated by this method in the presence of various sulphonamides (Table 7) show that it is equally efficient in the three mixtures.

Determination of PAS-sulphonamide mixtures

A differential method, similar to that described for the estimation of mixtures of PAS and sulphetrone, may be applied to the determination of mixtures of PAS and the sulphonamides.

The 'total diazotizable substance' is determined by the naphthylethylenediamine method, the PAS is estimated independently by coupling with diazotized *p*-nitraniline, and the correction for the PAS contribution then calculated as described on p. 304.

Recoveries of 91–108% were obtained in the estimation of mixtures of PAS and sulphanilamide, sulphadiazine or sulphaguanidine.

SUMMARY

1. Methods for determining *p*-aminosalicylic acid (PAS) in body fluids are reviewed.

2. Convenient methods for estimating *p*-aminosalicylic acid in the presence of sulphetrone and other sulphone derivatives, using *p*-dimethylaminobenzaldehyde, and in the presence of sulphonamides by coupling with diazotized *p*-nitraniline, are described.

3. A differential method for the determination of mixtures of *p*-aminosalicylic acid and sulphetrone is described.

4. A similar method for the determination of mixtures of *p*-aminosalicylic acid and sulphonamide drugs is described.

I wish to thank Miss I. Secker for valuable technical assistance.

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Paper Chromatography of Porphyrins: some Hitherto Unrecognized Porphyrins and Further Notes on the Method

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Filter-paper chromatography, using a lutidine-water system, has been applied to the separation of the porphyrins by Nicholas & Rimington (1949). The R_F values of free porphyrins are inversely proportional to the number of carboxyl groups in the molecule; hence with the aid of a reference graph one may deduce from the R_F values obtained in any paper chromatographic analysis of a mixture of porphyrins what classes of porphyrins are present, e.g. dicarboxylic porphyrins, coproporphyrins or uroporphyrins.

The technique has proved of value in demonstrating the presence in normal urine of small quantities of uroporphyrin together with coproporphyrin (Nicholas & Rimington, 1949), and also in identifying the porphyrins of the 'pearl shells' *Pteria* (*Pinctada*) *radiata* and *Pteria vulgaris* as uroporphyrin and coproporphyrin (Nicholas & Comfort, 1949). No support was obtained from paper chromatograms for the existence in marine molluscan shells of the pentacarboxylic porphyrin, termed 'conchoporphyrin' by Fischer & Hofmann (1937).

When the total urinary porphyrins of some cases of acute porphyria were examined by this method, it was discovered that in addition to known porphyrins containing two, four or eight carboxyl functions, there were frequently present appreciable quantities of porphyrins giving discrete fluorescent spots, the R_F values of which indicated groups with three,

five, six or seven carboxylic functions in the molecule.

In the present paper we record the occurrence of new porphyrins, as judged by paper chromatograms, in a variety of materials which we have investigated. Some further notes upon paper chromatography are also included.

METHODS

Porphyrins were extracted from urines, faeces, etc., by the well known procedures. They were usually purified by chromatography in acid solution upon small columns of talc (Nicholas & Comfort, 1949), eluted by dilute NH_4OH and applied to the paper strips in this solvent. Esters were hydrolysed by contact with approx. 7 N-HCl for 36 hr. at room temperature, the acid removed by vacuum desiccation over KOH and the residue dissolved in 2 N-NH_4OH.

Filter-paper chromatography was performed using a lutidine-water system, and to provide markers a mixture of uroporphyrin I, coproporphyrin I and deuteroporphyrin IX was always placed upon the same strip adjacent to the unknown. The paper used was 'Whatman no. 1 for chromatography'. Better results were obtained when the chromatograms were run in an atmosphere of lutidine and water only, without NH_3 vapour as originally described (Nicholas & Rimington, 1949). The R_F values were, however, slightly lower.

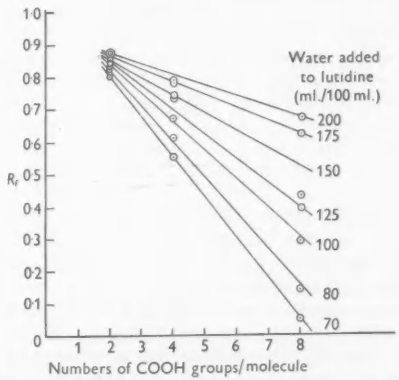

Fig. 1. Paper chromatography of porphyrins with different proportions of water to lutidine. Temp. 5°. Effect of quantity of water present upon R_F.

Effect of temperature. Equally good results are obtainable using lutidine saturated with water at temperatures varying from 18 to 25° and a similar ambient temperature during the run. The R_F values become lower with increase of temperature, but the linear relationship between the number of carboxyl functions and R_F still holds. The R_F value appears to depend upon the proportion of water present in the solvent system.

Effect of water content. Lutidine (a mixture of 2:4- and 2:5-dimethylpyridines) and water become completely miscible at a temperature of 17° and below. Good chromatograms may be run with the solvent not completely saturated with water, and experiments were carried out at 5° to determine the R_F values obtaining with lutidine-water mixtures of known composition (see Fig. 1). The linear relation between R_F and number of carboxyl functions holds good over the range of lutidine-water mixtures studied and, in the case of uroporphyrin at any rate, R_F appears to be linearly related to the quantity of water present.

At a temperature of 5° and with lutidine containing 40% (v/v) of water, the R_F values obtained on paper chromatograms are the same as those obtained at 21° with the water-saturated solvent. The lutidine-water phase at this higher temperature contains, however, the same proportion of water, namely, 40% (v/v).

In the present study, a temperature of $21 \pm 1°$ has been used and lutidine saturated with water at this temperature.

Effect of light. The best chromatograms with porphyrins are obtained by running them in the absence of light.

RESULTS

A summary of our findings is presented in Table 1.

DISCUSSION

It is clear from Table 1 that hitherto unrecognized porphyrins occur frequently in pathological and in some normal materials. The technique of paper chromatography has, in fact, revealed an 'intermediary metabolism of the porphyrins' which was unsuspected. The isolation of those pigments which stand midway between uroporphyrin with eight carboxyl groups and coproporphyrin with four carboxyl groups, and the determination of their exact structure, may be expected to throw further light upon the relation of the copro- and uroporphyrins to one another and to the erythropoietic system in general. We have commenced this task of isolation, using standardized methods of adsorption chromatography (Nicholas, 1951).

Indications have not been lacking in previous work that unidentified porphyrins existed. Thus, Watson (1935) isolated the crystalline ester (m.p. 202–203°) of a porphyrin resembling deuteroporphyrin from the faeces of a case of familial haemolytic jaundice; Grinstein, Schwartz & Watson (1945) claimed to have separated the Waldenström ester from urines of acute porphyria patients into uroporphyrin I and a heptacarboxylic porphyrin, presumably belonging to the isomeric series III; McSwiney, Nicholas & Prunty (1950) found in the faeces of a case of acute porphyria a porphyrin which upon chromatography displayed evidence of five carboxyl groups. More recently, Grinstein, Wikoff, de Mello & Watson (1950) have drawn attention to the presence of a porphyrin, which they term porphyrin *x* (ester m.p. 210–215°), in the faeces of rabbits experimentally poisoned by lead. Our finding of a tricarboxylic porphyrin in lead poisoning is interesting in this connexion.

The presence of a pentacarboxylic porphyrin, together with coproporphyrin in calf meconium, is

Table 1. *Partition chromatographic analysis of porphyrins present in some natural and pathological materials*

Material	$(COOH)_2$	$(COOH)_3$	$(COOH)_4$ (coproporphyrins)	$(COOH)_5$	$(COOH)_6$	$(COOH)_7$	$(COOH)_8$ (uroporphyrins)
Urine of ground squirrel (*Sciurus niger*)	.	+ +	(+)	(+)	.	.	+ + +
Meconium of calf	.	.	+ + +	+	.	.	.
Meconium of rabbit (*Lepus cuniculus*)	.	.	+	.	.	.	.
Meconium of civet cat (*Civettictis civetta*)	.	.	+	.	.	.	.
Meconium of bactrian camel (*Camelus bactrianus*)	.	(+)	+	.	.	.	.
Meconium of sea lion (*Zalophus californianis*)	.	.	+	.	.	.	.
Meconium of human baby	.	.	+	+	(+)	.	.
Urine from case of industrial lead poisoning	.	+	+ +	.	.	.	+
Coproporphyrin preparation from urine of lead-poisoned rabbits	.	+	+ +	.	.	.	.
Urine of acute porphyria case 'Glasgow'	+	.	.	+	.	+ +	+ + +
Urine of acute porphyria case 'Liverpool'	.	(+)	.	.	+	.	+ +
Urine of acute porphyria case 'S'	.	.	.	+	.	(+)	+ +
Urine of acute porphyria case 'Weaver'	.	.	.	.	.	.	+ +
Porphyrin methyl ester preparation, m.p. 143–162°, from case 'C' of porphyria cutanea tarda (Gray, Rimington & Thomson, 1948)	.	+	+ +	.	+	.	.
Urine from case 'L.W.' of porphyria cutanea tarda	+	+	+	+	.	+	+ +
Faeces from same case	.	+	+ +	.	.	.	(+)
Urine from case 'W' of porphyria cutanea tarda	.	.	.	.	(+)	+	+ +

Very strong spot, + + +; strong spot, + +; moderately strong spot, +; faint spot, (+).

of no little interest. Such pigments must definitely be products of the foetus and cannot have arisen as the result of bacterial activity in the gut. We have isolated this pentacarboxylic porphyrin and will shortly report upon its constitution. It may be recalled that Grinstein *et al.* (1945) encountered a pentacarboxylic porphyrin among the products of the decarboxylation of the Waldenström ester. They give a carbon and hydrogen analysis of the pentamethyl ester, which had m.p. 221–224° and the α-band in chloroform at 623·5 mμ. Further decarboxylation with hydrochloric acid at 180° led to coproporphyrin I.

SUMMARY

1. A further study has been made of the technique of paper chromatography of the porphyrins.

2. The method has been applied to the examination of the porphyrins present in the meconium of several animal species, urines in industrial and experimental lead poisoning and the urine and faeces from a number of cases of porphyria.

3. The frequent presence has been revealed of hitherto unrecognized porphyrins possessing three, five, six or seven carboxylic functions as determined by their chromatographic behaviour.

We wish to thank the Medical Research Council for a personal grant which supported this investigation, and Miss A. Benson, Mr P. Bliss and Miss P. A. Miles for their valuable assistance.

We are greatly indebted to Dr R. E. Rewell and Dr W. E. Lawrence of the Zoological Society of London for kindly placing at our disposal the specimens of animal meconium. The porphyrin from the urine of lead-poisoned rabbits was kindly supplied by Dr M. Weatherall of the London Hospital Medical College.

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Chromatographic Methods for the Separation and Identification of Porphyrins

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Reproducible results by chromatographic methods can only be ensured by strict adherence to experimental conditions. Although successful separations of porphyrin esters have been reported by previous workers using columns of alumina (Prunty, 1946; McSwiney, Nicholas & Prunty, 1950; Lederer & Tixier, 1947) or of calcium carbonate (Grinstein, Schwartz & Watson, 1945; Watson, Schwartz & Hawkinson, 1945; Gray & Holt, 1948), the adsorptive power of the commercial materials employed has not been strictly defined and, moreover, little if any reference has been made to the purification or standard of purity of the solvents employed. Repetition of their results, therefore, has often proved difficult.

Filter-paper chromatography has been applied to the porphyrins by Nicholas & Rimington (1949) and in the lutidine-water system used is capable of separating them into groups corresponding to the number of carboxyl functions in the molecule. It can thus be used as an analytical procedure, but appears to be not so well adapted for the preparation of material in quantities suitable for chemical characterization. For this purpose, adsorption chromatography of porphyrin esters upon solid columns is preferable.

Before attempting the isolation of certain hitherto unrecognized porphyrins, which partition chromatography has shown to be present in pathological materials (Nicholas & Rimington, 1951), we deemed it essential to study carefully the chromatographic behaviour of known porphyrin esters in precisely defined systems and to extend, if possible, the range of adsorbents available. This we have done, and our results are presented below. Paper chromatography has proved a very effective means of ascertaining the homogeneity of fractions, and therefore the sharpness of the separations obtained.

METHODS

Adsorbents

The commercial materials used were:

Al_2O_3, 'Aluminium oxide for chromatographic analysis standardized according to Brockmann', grade I (Savory and Moore Ltd.).

MgO, heavy magnesium oxide (British Drug Houses Ltd.).

$MgCO_3$, heavy magnesium carbonate (British Drug Houses Ltd.).

$CaCO_3$, calcium carbonate, 'Analar' (British Drug Houses Ltd.).

From these materials, adsorbents of graded adsorptive power were prepared as described below, and all preparations were finally standardized according to the technique of Brockmann & Schodder (1941), whose system of classification for aluminas we have extended to all our materials. Brockmann & Schodder's classification is summarized by Williams (1946).

Graded aluminium oxide. The Al_2O_3 supplied by Savory and Moore (see above) is termed by the manufacturers 'Grade I Brockmann', but we have found that samples can vary from grade I to grade II. The adsorptive power appears to depend upon the amount of water present. Samples can be made more active by heating followed by cooling in a confined atmosphere. Conversely they may be deactivated by the addition of water. Grade II Al_2O_3 can be prepared from any other grade by suspending in water, filtering, drying at 120° and then cooling in a desiccator. Grade IV Al_2O_3 was similarly prepared by wetting followed by drying thin layers in air at 20°.

Graded magnesium oxide. The adsorptive power of the commercial material is rather variable. The grades used in our study were prepared as were the aluminium oxides, the conditions of drying being as follows: grade II, dried at 120° and cooled in a confined space; grade III, dried at 37° in thin layers in a constant temperature room; grade IV, dried in thin layers in air at 20°.

Graded magnesium carbonate. Heavy $MgCO_3$ as supplied by British Drug Houses was found to be grade III. It behaves chromatographically in all respects as does MgO of similar grade.

Graded calcium carbonate. Samples of $CaCO_3$ from several different sources, when standardized, all conformed to grade V. They were improved, however, by a preliminary heating to 120° followed by cooling in a desiccator before use.

Adsorption columns

For most experiments, glass tubes were used of size 1·5 × 20 cm., tapering sharply at one end to about 3 mm. diameter, and provided with a light plug of cotton wool to support the adsorbent. They were filled to a height of 10 cm. with adsorbent in the following manner. The lower end was closed by a stopper and the glass tube almost filled with the solvent in which the adsorbent column was to be packed. The adsorbent was then sprinkled into the solvent until the tube was half filled. The stopper was removed and the column allowed to drain under gravity or, if necessary, gentle suction. In this way the adsorbent particles became completely wetted and produced the most evenly packed columns. This method was used for all adsorbents except $CaCO_3$. With this adsorbent the best results are obtained by first making a slurry with the solid and the solvent and then pouring it into the glass tube. Alternatively, the adsorbent may be packed tightly into the column in small portions at a time, using a tamping rod, and then wetted with the solvent. Although $CaCO_3$ is a very useful adsorbent for porphyrins, it is the most tedious to use owing to this difficulty in packing.

Solvents

The following solvents have been used, details of purification, where necessary, being given below.

Benzene. 'Analar' (British Drug Houses Ltd.). Suitable for use without further treatment; drying and redistilling did not affect its chromatographic behaviour.

Chloroform. The B.P. grade, containing ethanol, possesses a considerably greater eluting power than does the pure solvent. For use it was washed five times with 2 vol. of water and dried over $CaCl_2$ (see Vogel, 1948). It is then unstable and must be kept in the dark.

Light petroleum. The commercial product, b.p. 40–60°, was redistilled within these limits.

Methanol. The solvent was dried by the magnesium and iodine method according to Vogel (1948).

Application of porphyrin esters and development of chromatograms

Porphyrin esters (approx. 0·3 mg. of each) were applied to the columns as a concentrated solution in the first-named solvent of each system studied (see Fig. 1), the adsorbent having been packed suspended in this solvent. Development was accomplished by the mixture. Further details are included under the Results section. It may be noted here that in each case the last-named solvent in a system is that possessing the greatest eluting power. All chromatograms were run at room temperature, 19–22°.

Examination of fractions

Absorption spectra of fractions eluted from the columns were measured on the Beck-Hartridge reversion spectroscope.

Melting points were observed upon an electrically heated micro-melting point apparatus (Gallenkamp).

Partition chromatography was carried out as described by Nicholas & Rimington (1949, 1951) at a controlled temperature, 21 ± 1°. Porphyrin esters, eluted from the columns, were hydrolysed by evaporating the volatile solvent under reduced pressure and leaving the residue in contact with approx. 7 N-HCl at room temperature for 36 hr. Excess acid was removed in a vacuum desiccator containing KOH, the porphyrin hydrochloride dissolved in 2 N-NH_4OH and applied to the paper (Whatman, 'no. 1 for Chromatography').

RESULTS

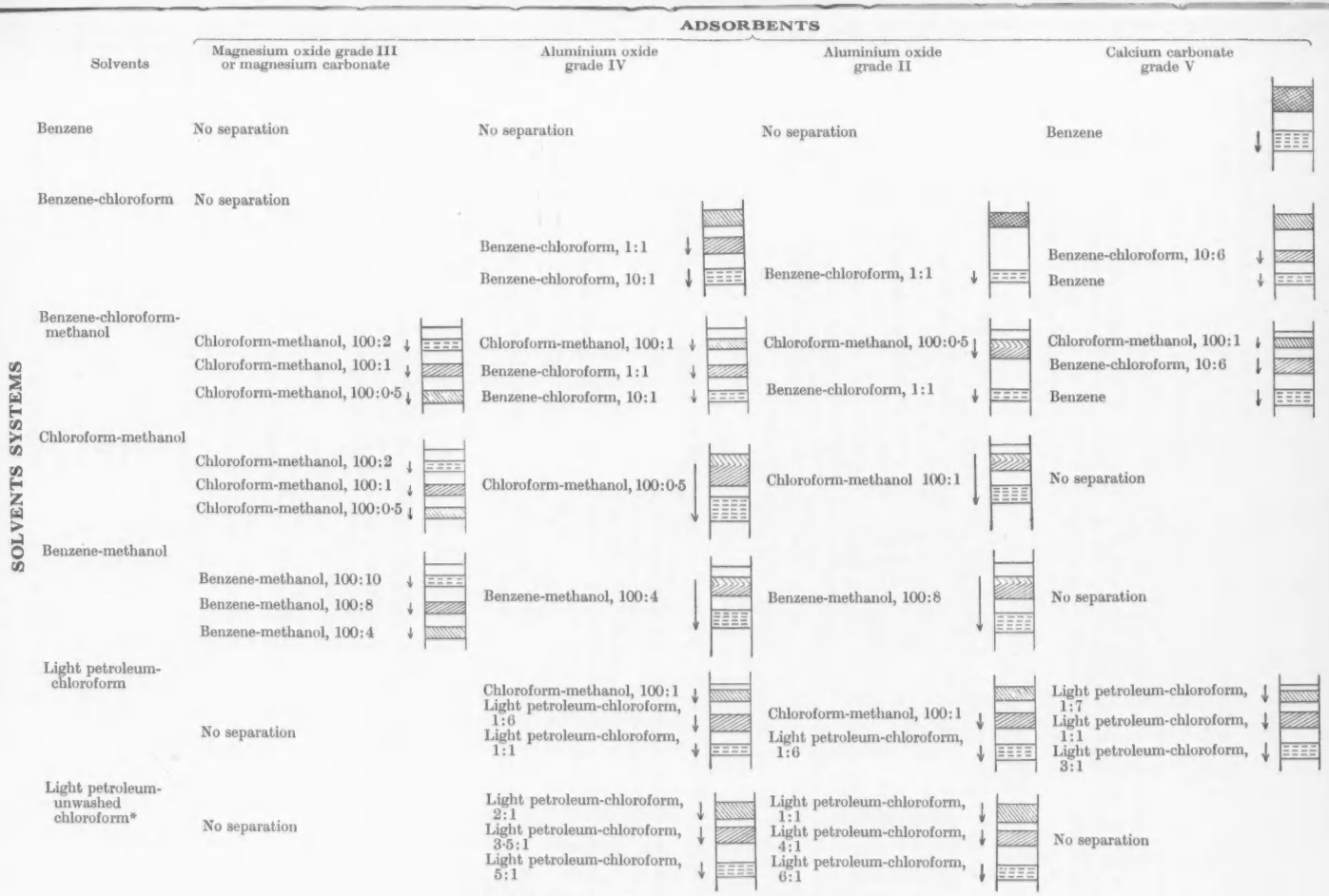
A schematic survey of our results is presented in Fig. 1. It was not possible to separate satisfactorily the I and III series isomers of either uroporphyrin or coproporphyrin with any combination of solvents and adsorbent tried. In general, porphyrin esters are eluted in an order corresponding inversely with the number of carboxyl groups present in the parent porphyrin. The uroporphyrins are thus the most firmly retained. From magnesium oxide and magnesium carbonate, however, the order is exactly reversed, the uroporphyrins being the first to leave the column. The dicarboxylic porphyrin esters all behave very similarly under the solvent conditions which separate them from uroporphyrin and coproporphyrin. Their individual separation is, however, possible and will be discussed in a separate section below.

Magnesium oxide and magnesium carbonate, grade III. Neither benzene nor chloroform possessed any appreciable eluting power with these adsorbents and no separation of esters was observed with mixtures ranging from 100 % benzene to 100 % chloroform. Upon the addition of methanol to the system, elution began, uroporphyrin running the most rapidly. In practice, therefore, the mixed esters may be applied in either benzene or chloroform, and development carried out progressively with mixtures of 0·5, 1 and 2 % (v/v) methanol-chloroform or 4, 8 and 10 % (v/v) methanol-benzene, respectively, which carry down the successive bands.

No satisfactory separation was achieved with solvent systems composed of light petroleum and chloroform, or those with methanol (or unwashed chloroform) as used by McSwiney *et al.* (1950).

Aluminium oxide, grade II. The solvent systems benzene-chloroform, benzene-methanol and chloroform-methanol produced separation of the dicarboxylic porphyrin esters as a discrete band below the uroporphyrin and coproporphyrin esters; these formed two contiguous bands which were inseparable and travelled down the column together.

Aluminium oxide, grade IV. The benzene-chloroform solvent system gave with this weaker adsorbent separation of the three groups of esters, coproporphyrin and the dicarboxylic porphyrin esters being eluted without the addition of methanol. Uroporphyrin was eluted when the latter solvent was added to the system. With chloroform-methanol and benzene-methanol, however, this adsorbent



* Equivalent to chloroform containing 1% (v/v) methanol.

Fig. 1. Adsorption chromatography of porphyrin methyl esters. ▨, uroporphyrins; ▨, coproporphyrins; ⋮⋮, dicarboxylic porphyrins; ↓ indicates movement of band on columns.

behaved in the same way as grade II aluminium oxide.

In the case of light petroleum-chloroform or the same system plus methanol, good separation of the porphyrin esters is produced with both grades of alumina (cf. McSwiney *et al.* 1950). The procedure is somewhat complicated since progressive solution of the porphyrin components takes place with increasing concentration of chloroform in the developing solvent. Fig. 2 illustrates the separation.

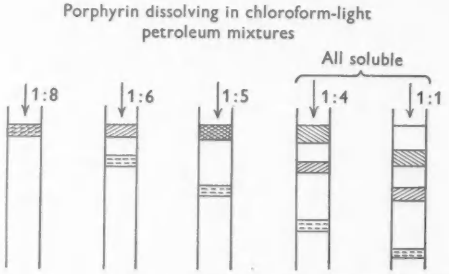


Fig. 2. Chromatography of esters on aluminium oxide grade II using chloroform-light petroleum mixtures. Ratio vol./vol. shown on figure. ▧, uroporphyrins; ▨, coproporphyrins; ⁙, dicarboxylic porphyrins.

Calcium carbonate, grade V. Development by benzene of a chromatogram of the porphyrin esters on this adsorbent causes the dicarboxylic porphyrin esters to separate from the remainder as a mobile band. Addition of chloroform (6 vol. chloroform to 10 vol. of benzene) causes migration of the coproporphyrin band. Uroporphyrin ester is only mobilized by the addition of methanol (1 vol. methanol to 100 vol. of chloroform). If benzene-methanol or chloroform-methanol systems are used from the start, elution takes place without separation.

With a technique similar to that mentioned above for alumina, in which the porphyrins are applied to the column in light petroleum-chloroform, separation of the three classes of esters is possible.

Separation of the dicarboxylic porphyrin esters

In these experiments the esters of protoporphyrin, deuteroporphyrin and mesoporphyrin have been employed. On account of the close chemical similarity between these pigments, chromatographic separation has presented more difficulties than when dealing with porphyrins containing differing numbers of carboxyl groups. Of the three, protoporphyrin is the most firmly adsorbed, no doubt on account of its two vinyl groups. Either magnesium oxide, grade III, or calcium carbonate, grade V, may be used for separation (see Fig. 3).

Magnesium oxide, grade III. The esters are applied in chloroform solution and development performed with mixtures of 0·5:100, 2:100 and 3:100 (v/v) methanol-chloroform used successively. Mesoporphyrin, deuteroporphyrin and protoporphyrin esters are eluted in that order.

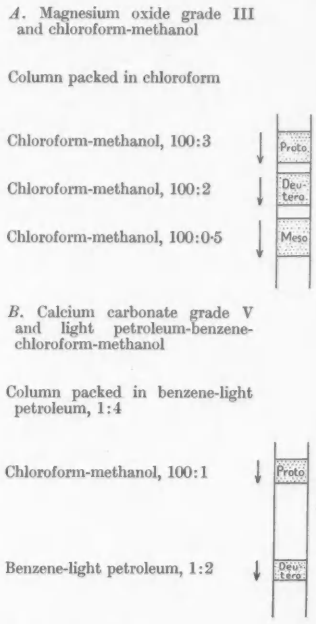


Fig. 3. Separation of dicarboxylic porphyrin esters.

Calcium carbonate, grade V. The esters are applied dissolved in benzene-light petroleum, 1:4 (v/v). For development the benzene is then increased to 2:1 (v/v), at which stage deuteroporphyrin ester passes down the column. Protoporphyrin ester is eluted with methanol-chloroform, 1:100 (v/v).

DISCUSSION

Magnesium oxide preparations constitute a valuable addition to the technical resources of porphyrin chromatography. The columns are easily packed, percolation by solvents is rapid and separations are good. In addition, the uroporphyrins are the first to be eluted from this adsorbent and its choice is therefore indicated for preparative purposes when these porphyrins are the main objective.

The separation of the naturally occurring dicarboxylic porphyrins upon magnesium oxide or calcium carbonate is a useful technical device. The combined dicarboxylic fraction may be obtained in

a primary chromatogram and then resolved by a secondary chromatogram upon one or other of these adsorbents. Filter-paper chromatography, it may be recalled, does not differentiate between the members of this group (Nicholas & Rimington, 1949).

It is unfortunate that none of the systems we have studied has been capable of separating sharply the I and III series porphyrin isomers. It may be noticed, in particular, that we failed to separate uroporphyrin esters I (from bone) and III (Waldenström ester), whereas Fischer & Hofmann (1937) claimed to have done this by chromatographing uroporphyrin ester from Petry urine upon talc. They ascribed to uroporphyrin ester I a melting point of 311° (corr.) and to the series III isomer a melting point of 261° (uncorr.), but Grinstein *et al.* (1945) have cast serious doubt upon this claim. The material with m.p. 311° is thought by them to have been a uroporphyrin metal complex (probably copper) and the existence in nature of a uroporphyrin belonging to the aetioporphyrin III series is considered unproven. The Waldenström ester is thought by Grinstein *et al.* (1945) to be a mixture or molecular compound of uroporphyrin I with a heptacarboxylic porphyrin (probably of series III).

In a later paper the application of chromatographic methods to the isolation of new porphyrins from various sources will be described.

SUMMARY

1. The chromatographic behaviour has been studied of porphyrin esters under standardized conditions.

2. Magnesium oxide and magnesium carbonate possess certain advantages for porphyrin chromatography.

3. The naturally occurring dicarboxylic porphyrins may be separated chromatographically upon calcium carbonate or on magnesium oxide columns.

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Displacement Chromatography on Synthetic Ion-exchange Resins

6. EFFECT OF TEMPERATURE ON THE ORDER OF DISPLACEMENT

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During the course of development of the work described in this series of papers it has become increasingly clear that non-ionic adsorption effects play an important part in determining the order of displacement of bases and ampholytes from columns of synthetic cation exchangers. In Part 4 of this series (Partridge, 1949*b*) the various factors then known to affect the order of displacement were discussed in some detail. Previously Davies (1949) had shown, from theoretical considerations, that an ideal cation-exchange process would be expected to result in the separation of a series of monoamino-acids in an order depending upon their pK_1 values; that is to say, upon the negative logarithms of the acid dissociation constants for the reaction: $A^+ \rightleftharpoons A^{\pm} + H^+$. For the purposes of illustration Davies assumed a

constant and low pH at the surface of the resin throughout the major part of the separation, but his arguments apply equally to the case where a mixture of monoamino-acids, free from mineral acid, is applied to a column packed with a strongly acidic cation-exchange resin in its hydrogen form.

As pointed out by Davies, the order of displacement from the sulphonated phenol-formaldehyde resin Zeo-Karb 215 at room temperature (Partridge, 1949*a*) does in fact reflect the order of pK_1: but there are certain exceptions. Thus, valine (pK_1, 2·32) and proline (pK_1, 1·99) form a mixed band and are displaced by a mixed band containing leucine (pK_1, 2·36) and methionine (pK_1, 2·28). Another exception concerns cystine (pK_1, 1·0; pK_2, 2·0), the behaviour of which is complicated by its capacity to form a bivalent cation. It seems probable that the high affinity of methionine for the resin is due to a strong contribution from van der Waals forces since methionine is adsorbed more strongly than leucine on charcoal (Tiselius, 1947).

Changes in the temperature at which a displacement experiment is carried out would be expected to modify the system in a complex way. Thus the dissociation constants both of the insoluble resin acid itself and of the ionizable solutes in the liquid phase would be altered. In addition, the van der Waals contributions and the degree of hydration of the resin and the solutes in the liquid phase would also be expected to change.

Measurements of pK_1 for various amino-acids over a temperature range up to 50° are available (Cohn & Edsall, 1943), and from these it appears that changes in temperature over this range do not greatly affect the relative values. It may thus be assumed that relative changes in the pK_1 values of the amino-acids due to increasing temperature are unlikely to be large enough to result in changes in the order of displacement.

The effect of hydration on the ion-exchange behaviour of a series of inorganic cations on natural zeolites has been discussed by Taylor & Urey (1938), who point out that any change in conditions affecting the solvation of the ions, such as change in the nature of the solvent or temperature, changes the behaviour of the ions. Similar considerations are applicable to the ion-exchange behaviour of organic ampholytes, and it may well be that the anomalous behaviour of certain of the amino-acids is due to the effects of hydration. Thus, proline is unique among the amino-acids in its high solubility in both water and ethanol, and its anomalous ion-exchange behaviour may be due to a high degree of solvation in aqueous solution.

These considerations have led us to attempt to resolve certain of the mixed bands reported in Part 3 of this series (Partridge, 1949*a*) by carrying out a further fractionation using the same resin but at a higher temperature. An increase in temperature would be expected to provide a situation in which the order of displacement of the amino-acids follows that suggested by Davies more closely, and should thus result in the separation of proline from valine and methionine from leucine.

EXPERIMENTAL AND RESULTS

Materials

The sulphonated-polystyrene resin used in these experiments was prepared in the laboratory following the procedure given by Partridge, Brimley & Pepper (1950). A lightly cross-linked product was used, the nominal divinylbenzene content of the mixture of monomers, before polymerization, being 4% (w/w). The spherical particles of resin were graded by sieving in the air-dry condition before use, and in the experiments described here the columns were packed with the 40–70 mesh/in. fraction.

Zeo-Karb 215, as received from the makers (Permutit Company, London), was ground in a hammer mill and graded by sieving in the air-dry condition. The sieved powder was allowed to stand overnight in 5N-HCl and was then thoroughly washed in an upward flow of water in order to remove fine particles. The columns were packed with the washed resin and treated alternately with N-NH_3 and 2N-HCl several times in order to complete the removal of impurities from the resin and to compact the bed.

Apparatus

The construction and packing of the columns was generally as given in Part 1 of this series (Partridge & Westall, 1949). However, since it has been shown (Hagdahl, 1948; Partridge, 1950) that considerable improvement in the sharpness of separation is obtained by use of a system of coupled columns of diminishing diameter, this device has been employed for both the experiments at room temperature and those carried out at higher temperatures.

For small-scale work, where columns 1·5 cm. or less in diameter were employed, it was found that steady temperatures up to 90° could be maintained by enclosing the whole column assembly in an air jacket. In the apparatus used, air was continuously circulated, by means of a rotary blower, through a closed system consisting of the column jacket and a small electric furnace. Temperature was controlled to $\pm 2°$ by regulating the output of the furnace by means of an external resistance.

The use of an air jacket in this way has the merit of simplicity, but is unsuitable for large-scale work involving large-diameter columns and high rates of liquid flow. For this purpose it is necessary to pre-heat the inflowing liquid as well as to maintain the temperature of the surfaces of the columns and connecting tubes. A water-jacketed column appeared to offer the simplest arrangement, and Fig. 1 shows a jacketed column constructed for operation at temperatures up to 60°. The column consisted of three sections (*A*, *B*, *C*) connected in series one above the other. The sections were of diameter 5, 3·3, 2·3 cm., and height 50, 22·5, 12·5 cm., respectively, and each was packed with resin to about 4 cm. from the open top. Each section was provided with a float (*D*, *E*, *F*), made from disks of 'polythene', in order to prevent disturbance of the top surface of the resin by the jet of liquid flowing into the

section. The jacket (*G*) was made from a length of industrial glass pipeline with flanged ends. Standard flanged joints were used at the top and bottom of the jacket in order to bolt on the metal faceplates (*H*, *I*). The jacket was made watertight by employing thick rubber gaskets under both faceplates.

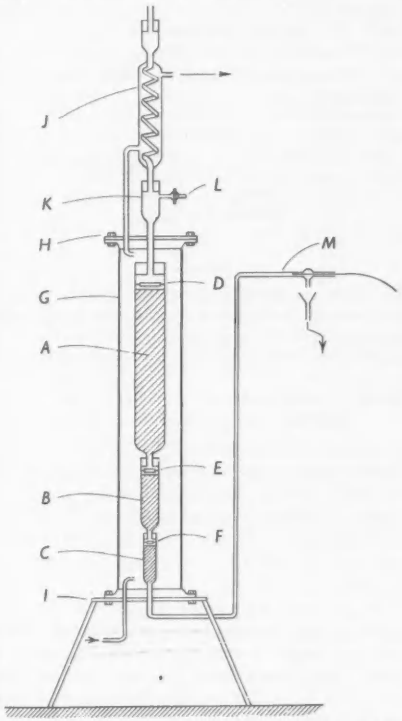

Fig. 1. Jacketed column assembly for use at high temperatures, showing set of three coupled columns and flow regulator. A description is given in the text.

Liquid entering the column passed first through a spiral condenser (*J*) which was heated by water from the jacket. The pre-heated solution passed to the column through the air trap (*K*), air being released from time to time by means of the tap (*L*). The rate of flow through the column was regulated by the valve (*M*) which consisted of a piece of stainless steel wire inserted into a closely fitting glass capillary carrying a bulb and side arm near the open end. It was found that the rate of flow could be adjusted accurately by varying the depth to which the wire was thrust into the capillary.

Displacement of a mixture of leucine and methionine from sulphonated-polystyrene resin at room temperature, 60 and 80°

In order to avoid disturbances due to the marked swelling and shrinkage of the sulphonated cross-linked polystyrene (cf. Partridge *et al.* 1950) the column was constructed in two sections, the lower section being very short in relation to its diameter. Wall effects are reduced as the height of the resin bed decreases, and the provision of a short independent section at the lower end of the column tends to correct any boundary disturbance arising in the long upper section.

Displacement at room temperature. The main column was 27 cm. high (packed in a glass tube of 11 mm. bore), while the lower section was 2·5 cm. high (bore 13·5 mm.). The weight of resin (40–70 mesh/in.) packed into the two filtration tubes was 8·5 g. (dry wt., sodium form). A solution of L-leucine (10 mmol.) and DL-methionine (10 mmol.) made up to 130 ml. with water was passed through the column at a rate of 60 ml./hr. The column was rinsed with a little distilled water (10–20 ml.). This was followed by 0·20 N-sodium hydroxide solution (200 ml.) passed at a rate of 40 ml./hr. Both the lower amino-acid boundary and the upper boundary due to sodium hydroxide could be followed by observing the slight colour change in the light-coloured resin. The effluent solution was collected in 5 ml. fractions, and a drop from each fraction applied to the base line of a filter-paper partition chromatogram using butanol-acetic acid as the solvent. Fig. 2*a* is a diagrammatic representation of the resulting chromatogram, and shows that no separation was observed under these conditions.

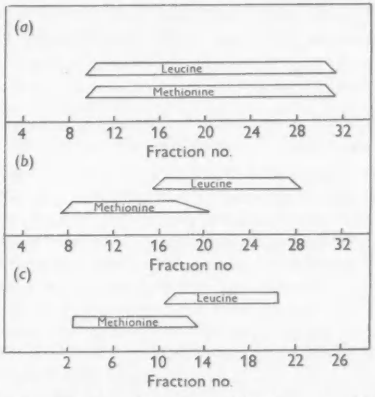

Fig. 2. Fractionation of a mixture of leucine and methionine by displacement from a column of sulphonated polystyrene (40–70 mesh/in.) with 0·2 N-NaOH. (*a*) Displacement at room temperature. (*b*) Displacement at 60°. (*c*) Displacement at 80°.

Separation of leucine and methionine at 60°. The column used in the experiment described above was regenerated with 2 N-hydrochloric acid and washed with distilled water. It was placed in an air jacket maintained at 60°. Fractions 9–31 from the previous experiment (Fig. 2*a*) containing the mixture of

leucine and methionine were mixed together and the solution (110 ml.) was heated to 100° for a few minutes in order to expel dissolved air. The solution was cooled to 60° and applied to the column at the same rate as before. The column, after washing with a little air-free distilled water, was displaced with 0·2 N-sodium hydroxide which had previously been heated to 100° and cooled to 60°. The sodium hydroxide solution was applied at a rate of 40 ml./hr. and the effluent collected in 5 ml. fractions. The results of a paper chromatogram of the fractions (Fig. 2*b*) showed that a clear separation was obtained.

Separation of leucine and methionine at 80°. The experiment described above was repeated under identical conditions except for an increase in the temperature of the jacket to 80°. The chromatogram is shown in Fig. 2*c* and indicates a rather sharper separation than that obtained at 60°.

The series of experiments described above show that although leucine and methionine may be separated quite readily at 60° they have no tendency to separate at room temperature. It is known that the boundaries of the bands are reduced in width as temperature increases (Partridge, 1949*c*) and indeed the boundary width observed at 80° was less than at 60°. However, the effect of increasing temperature cannot be explained on this basis alone, since, as the next section shows, leucine and methionine form a mixed band which separates from valine at room temperature, while at higher temperatures valine and methionine form a mixed band which separates from leucine.

Separation of a mixture of valine, leucine and methionine at room temperature and at 60°

The column used was the same as that employed in the previous series of experiments. A solution (120 ml.) containing DL-valine (8 mmol.), L-leucine (8 mmol.) and DL-methionine (8 mmol.) was applied to the column and the mixture displaced with 0·2 N-sodium hydroxide, the rate of flow being adjusted to 40 ml./hr. The effluent was collected in 5 ml. fractions. A paper chromatogram using butanol-acetic acid as the solvent was prepared as before, but since, in this solvent, the R_F value of methionine (0·47) lies close to that of valine (0·50), each spot, after application to the base line of the chromatogram, was treated with a drop of hydrogen peroxide solution in order to convert the methionine to its sulphone (Dent, 1948).

The result of the experiment at room temperature is shown in Fig. 3*a*. No separation was observed between leucine and methionine, but there was a partial separation of valine which tended to be displaced by the other two components of the mixture. The experiment was repeated under identical conditions but with a jacket temperature of 60°. The analysis of the effluent solution (Fig. 3*b*) showed that at 60° valine and methionine formed a mixed band and were displaced by leucine.

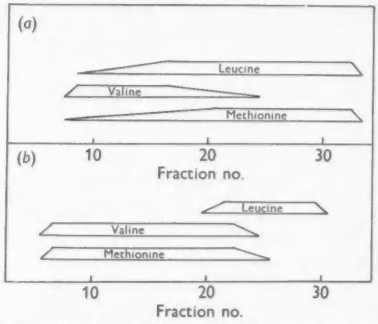

Fig. 3. Fractionation of a mixture of leucine, valine and methionine by displacement from a column of sulphonated polystyrene (40–70 mesh/in.) with 0·2 N-NaOH. (*a*) Displacement at room temperature, (*b*) displacement at 60°.

Isolation of methionine and a mixture of the isomeric leucines from a protein hydrolysate

A number of the experiments described above were repeated using a commercial sulphonated phenol-formaldehyde resin, Zeo-Karb 215, in place of the laboratory-prepared sulphonated polystyrene. The results were essentially the same, and it proved more convenient to use the commercial resin for larger scale work.

The primary fractionation of the hydrolysis product of egg albumin has already been described (Partridge, 1949*a*, 1950). The starting material used in this experiment was the leucine-methionine fraction taken from a primary fractionation carried out with the hydrolysis product from 232 g. (dry wt.) of commercial egg albumin. The solution (1500 ml.) contained leucine, isoleucine, methionine and a little cystine and was approximately 0·10 M with respect to the amino-acids. The column used was that illustrated in Fig. 1; a full description has already been given (p. 314).

The solution containing the mixture of amino-acids was filtered to remove a deposit of cystine; it was then brought to the boil and cooled to 60°. The jacket temperature of the column was adjusted to 56° and the solution passed through at a rate of 500 ml./hr. This was followed by about 50 ml. of distilled water to rinse the reservoir and connecting tubes. Ammonia solution (0·16 N, 10 l.) was then passed to the column at 500 ml./hr., the effluent solution being collected in fractions of 45 ml. by means of an automatic apparatus. Fig. 4 shows the analysis of the fractions.

Recovery of L-*methionine*. Fractions 4 and 5 (Fig. 4) were mixed, concentrated to about 50 ml. and allowed to stand at

0° for 2 or 3 days. A small crop of cystine was removed, and the filtrate treated with washed charcoal until free from cystine. The filtrate was then added to fractions 1–3 (Fig. 4) containing methionine only, and the whole was concentrated to 90 ml. Hot ethanol (90 ml.) was added and the L-methionine crystallized in fine needles (2·44 g.). Two further crops were obtained by further evaporation and the addition of ethanol. Total yield, 3·46 g. (Found N, 9·5. Calc. for $C_5H_{11}O_2NS$: N, 9·4%); $[\alpha]_D^{20°}$ +19·11° (*c*, 3·3 in 3 N-HCl; 2 dm.). Paper chromatograms carried out on the recovered methionine showed it to be free from other amino-acids. The recovery represented a yield of 1·5% calculated on the dry weight of protein taken. This figure is much lower (as judged by an analysis of pure ovalbumin; Chibnall, 1945) than can be accounted for by losses during the chromatographic procedure, and it is thought that serious loss may have

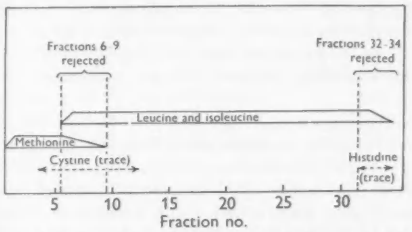

Fig. 4. Isolation of L-methionine and the isomeric leucines from the hydrolysis product of egg albumin. Displacement of band V (from the primary fractionation) by means of 0·16 N-NH_3 at 55°. Column packed with Zeo-Karb 215.

occurred during the hydrolysis of the impure protein. Commercial egg albumin contains a considerable amount of carbohydrate which may give rise to loss of methionine by side reactions of the type investigated by Lugg (1938; cf. Mohammad, Fraenkel-Conrat & Olcott, 1949).

Recovery of the mixture of leucine and isoleucine. Fractions 10–12 (Fig. 4) contained a little cystine in addition to leucine and isoleucine. They were mixed and concentrated by evaporation, when a small crop of cystine was deposited on standing. This was removed and the filtrate treated with successive small quantities of washed charcoal until a paper chromatogram showed the filtrate to be free from cystine. The filtrate was then added to the mixed solution from fractions 13–31 (Fig. 4), and the whole evaporated under reduced pressure to the crystallization point. An equal volume of hot ethanol was then added and the mixture of leucines allowed to crystallize. A second crop was obtained by further evaporation and the addition of ethanol. Total yield 18·8 g. (Found N, 10·7. Calc. for $C_6H_{13}O_2N$: N, 10·7%.) The yield represented a recovery of 8·2% calculated on the dry weight of protein taken; the mixture of leucines was shown, by paper chromatography, to be free from other amino-acids.

Isolation of L-*valine and* L-*proline from the hydrolysis products of a protein*

The primary separation of the products of hydrolysis of commercial egg albumin (dry wt. 64 g.) was carried out as previously described (Partridge, 1949*a*). The fractions containing valine and proline (band IV) were collected together so as to include smaller amounts of glycine and alanine from the overlapping boundary of band III together with a little leucine and isoleucine from band V. The solution was refractionated at room temperature, using a smaller column of Zeo-Karb 215 and following the details of procedure already given (Partridge, 1949*a*). The analysis of the effluent solution is shown in Fig. 5*a*. Fractions 9–23 (Fig. 5*a*) were mixed and

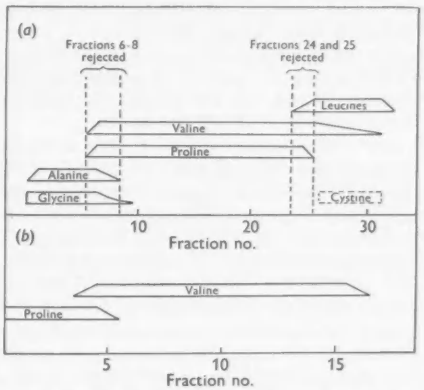

Fig. 5. Isolation of L-valine and L-proline from the hydrolysis product of egg albumin. (*a*) Displacement of band IV (from the primary fractionation) by means of 0·16 N-NH_3 from a column of Zeo-Karb 215 at room temperature. (*b*) Displacement of the mixture of valine and proline from experiment (*a*) (fractions 9–23) from a column of the same resin at 53°. Some of the proline was accidentally lost in this experiment.

the solution (after de-aeration by boiling) was refractionated on the same column at 60°, using 0·16 N-ammonia solution as the displacement developer. The resulting chromatogram (Fig. 5*b*) showed that, at 60°, valine was well separated from proline; some of the L-proline was accidentally lost during this experiment.

Recovery of L-*proline.* Fractions 1–3 (Fig. 5*b*) were collected and the solution evaporated to dryness under reduced pressure. The residue was dissolved in hot ethanol and the yellow solution decolorized with a little charcoal. Ether was added to the warm filtrate and the solution set aside to crystallize. Yield of L-proline, 0·66 g. (Found, N, 12·2. Calc. for $C_5H_9O_2N$: N, 12·2%) $[\alpha]_D^{20°}$ −84·0° (*c*, 1·22 in water; 2 dm.). The recovery represented 1·03% dry weight of protein taken.

Recovery of L-*valine.* Fractions 6–15 (Fig. 5*b*) were collected and the solution evaporated under reduced pressure until a few crystals appeared. An equal volume of hot ethanol was then added and the L-valine crystallized immediately in large plates. A second crop of crystals was obtained by further evaporation with the addition of ethanol. Total yield

1·8 g. (Found N, 11·9. Calc. for $C_5H_{11}O_2N$, 12·0%); $[\alpha]_D^{20°}$ +27·6° in 6·07 N-HCl (*c*, 3·33; 2 dm.). The recovery represented 2·8% dry weight of protein taken.

Separation of proline and valine: effect of reversing the order of the steps

The preceding section describes the isolation of proline and valine from a protein hydrolysate in the following steps: (1) The primary separation (Zeo-Karb 215 at room temperature) gave the proline-valine band heavily overlapped with glycine, alanine and leucine. (2) Fractionation using a narrower column of Zeo-Karb 215 at room temperature allowed a band consisting of proline and valine only to be isolated. (3) The proline was separated from the valine by fractionation at 60°.

It appeared that there would be some advantage in reversing the order of steps (2) and (3), but it could not be assumed that such a reversal of order would necessarily give satisfactory results since it has been observed that the separation of two components is frequently modified by the presence or absence of a third component of similar affinity for the resin. The point was therefore investigated experimentally.

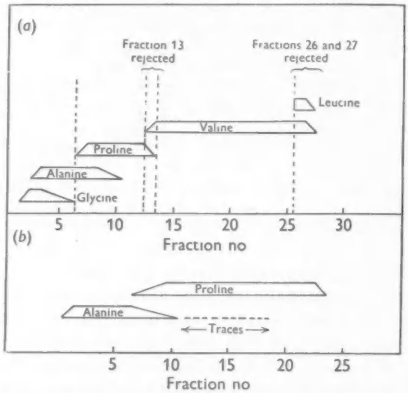

Fig. 6. Isolation of L-valine and L-proline from the hydrolysis product of egg albumin. (*a*) Displacement of band IV (from the primary fractionation) by means of 0·15 N-NH_3 from a column of Zeo-Karb 215 at 55°. (*b*) Displacement of a mixture of alanine and proline from experiment (*a*) (fractions 7–12) from a column of the same resin at room temperature.

The proline-valine band from a primary fractionation carried out with the hydrolysate from 232 g. (dry wt.) of commercial egg albumin was selected for the experiment. The solution was fractionated at 55° using 0·15 N-ammonia as the displacement developer. The analysis of the effluent solution is given in Fig. 6*a*. Fractions 14–25 (Fig. 6*a*), containing valine only, were collected and the valine recovered by crystallization. The yield of L-valine was 10·55 g.; N, 11·9%; $[\alpha]_D^{20°}$ +27·1° in 6·07 N-HCl (*c*, 3·43; 2 dm.). This represented a recovery of 4·54% calculated on the dry weight of protein taken, and the yield was thus much higher than that obtained in the previous experiment.

Fractions 7–12 (Fig. 6*a*), containing proline and alanine only, were collected and refractionated at room temperature by displacement from a column of the same resin. The analysis of the effluent (Fig. 6*b*) shows that although the bulk of the alanine separated from the proline as expected, the proline band was contaminated by traces of alanine which persisted almost throughout its length. Separations by displacement development are usually very sharp, and effects of this kind are rarely observed except in the case of cystine. However, the effect appeared to be real since when the proline and alanine solutions were mixed together and refractionated using a freshly packed column the result was identical.

It thus appears that proline and alanine may be separated quite readily at room temperature when in the presence of valine and glycine, but in the absence of these two amino-acids the separation is difficult. The effect is reminiscent of the formation of azeotropes in fractional distillation and a review of the separations, so far achieved by displacement from columns of synthetic ion exchangers, leads to the view that the formation of 'azeotropic mixtures' may to a large extent explain the sharpness of separation between mixed bands so frequently observed.

DISCUSSION

So far there have been few publications dealing with the effects of temperature on ion-exchange reactions. Nachod & Wood (1944) showed that, for a cation exchanger of the sulphonated coal type, there was little increase in the rate of exchange between calcium and hydrogen ions when the temperature was increased from 27 to 60°, although the reaction was slower at 0°. On the other hand, Ketelle & Boyd (1947), using sulphonated-polystyrene resins (Dowex 50), showed considerable improvement in the sharpness of separation between two rare earth elements on increasing the temperature of an experiment from 25 to 100°. As far as the resins that have been used here are concerned, some preliminary experiments carried out in this laboratory have shown that the boundary width given by sodium hydroxide solution on columns packed with a laboratory-prepared sulphonated-polystyrene resin (nominal divinylbenzene content, 4%) were reduced by approximately 40% as the temperature was raised from 17 to 36°. A similar reduction of the boundary width of ammonia fronts has been observed when columns packed with Zeo-Karb 215 were used at higher temperatures (Partridge, 1949*c*).

The undoubted effect of higher temperature in increasing the reaction rate of some ion-exchange resins may be employed usefully where the resins themselves are sufficiently stable to withstand decomposition, and increasing the temperature should result in a useful reduction in the time required to effect a given separation. In other cases, such as for instance the isolation of L-aspartic acid, L-glutamic acid or L-leucine, when the component has a low solubility in cold water, an increase in temperature may be desirable in order to allow the use of a displacement developer of higher concentration than would otherwise be possible. With aspartic acid, for instance, it is rarely possible to displace the amino-acid at a concentration greater than 0·1 M since it tends to crystallize out in the column. Higher concentrations of displacement developer could be used at higher temperatures, and this would avoid the removal of much water when it is required to recover the component by crystallization.

However, the results reported here show that there is a further effect of increasing the temperature, other than simply increasing the reaction rate. In some cases there is a qualitative alteration in displacement. Thus, Fig. 3*a* shows that leucine and methionine will together displace valine at room temperature, while at 60° (Fig. 3*b*), the valine forms a mixed band with methionine which is displaced by leucine. This shift in the position of methionine, together with a similar shift observed with proline, offers a ready means of isolating the four amino-acids, proline, valine, methionine and leucine, when present in mixed solution.

In general the position in the order of displacement taken up at higher temperature by an amino-acid approaches more closely the position expected from its dissociation constant for the process:

$$A^+ \rightleftharpoons H^+ + A^{\pm}.$$

Changes in position are not observed among the majority of the amino-acids for which the order of displacement is already qualitatively identical with the order of pK_1. Thus, when a mixture of mono-amino-acids from a protein hydrolysate (excluding the aromatic amino-acids) was displaced from a column of sulphonated-polystyrene resin at 60° the order observed was identical with that found at room temperature except for methionine and proline, which were both displaced more readily and appeared higher in the order. Various attempts to separate pairs of amino-acids of similar pK_1 value (such as glycine, pK 2·34 and alanine, pK 2·34) were as unsuccessful at 60 or 80° as at room temperature, in spite of the improvement in the efficiency of the column resulting from the increased temperature. It thus appears that increasing the temperature reduces the relative effect of adsorptive forces tending to increase the affinity of the amino-acid or its ion for the resin. The effect of such forces will change with changing environmental conditions and will be sensitive to temperature, both directly and indirectly, through changes in the state of hydration of the ions and the resin.

SUMMARY

1. Chromatographic displacement of mixtures of amino-acids from columns containing synthetic resinous cation exchangers gives rise to a number of mixed bands containing small groups of amino-acids which cannot be resolved by further treatment with cation-exchange resins at room temperature.

2. The effect of displacement at higher temperature has been investigated, and it is shown that this results in a reversal of the order of displacement affecting methionine and proline. Use is made of this in order to isolate L-proline, L-valine, L-methionine and a mixture of the isomeric leucines from a protein hydrolysate.

3. Two synthetic resinous cation exchangers have been investigated, a phenol-formaldehyde resin containing nuclear sulphonic acid groups (Zeo-Karb 215) and a sulphonated polystyrene resin (prepared in the laboratory). Similar results were obtained with each.

4. In discussing the mechanism it is shown that at higher temperature the order of displacement conforms more closely with that deduced from consideration of the pK_1 values of the amino-acids. Deviations from the ideal affecting methionine and proline at room temperature may be due, in large part, to the combined effects of hydration and van der Waals forces. As temperature increases, these deviations would be expected to diminish.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Synthesis and Secretion of Amylase by Pigeon Pancreas *in vitro*

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In this investigation a new approach has been made to the study of protein synthesis in animal tissues *in vitro*. An incorporation of labelled amino-acids into proteins has been shown by previous workers to occur in tissue homogenates and slices, and this has been regarded as evidence of protein synthesis *in vitro*. However, no net increase in proteins has so far been reported.* In planning the present experiments, it was thought that the chance of detecting a net increase of a protein would be good if digestive glands were used as the experimental material. These glands are equipped to synthesize enzymes, i.e. proteins, at rapid rates, and many of these enzymes can be quantitatively determined.

The first series of experiments on the formation of pepsin by gastric mucosa proved unsuccessful. The formation of amylase by pigeon pancreas slices was next studied, and this proved a suitable system. Under appropriate conditions the amylase activity increased by over 100% on aerobic incubation.

Part of this work has been communicated to the Biochemical Society (Hokin, 1950).

EXPERIMENTAL

Media. The following media were used: (1) the bicarbonate saline of Krebs & Henseleit (1932), gassed with either 5% CO_2+95% O_2 or 5% CO_2+95% N_2; (2) medium III (without the organic constituents) of Krebs (1950), gassed with O_2; (3) phosphate saline of Krebs & Eggleston (1940), gassed with O_2; (4) sheep serum (inactivated by heating at 60° for 2 hr.), gassed with 5% CO_2+95% O_2; the heat treatment destroyed the serum amylase activity. NaOH (0·2 ml. of a 10% (w/v) solution) was placed in the centre well when medium 2 or 3 was used. All media contained 0·2% (w/v) glucose. In some experiments either 'supplemented casein hydrolysate' or a mixture of amino-acids was added. The supplemented casein hydrolysate consisted of acid-hydrolysed casein prepared by the method of McIlwain & Hughes (1944) with 1·5 parts of L-tryptophan added per 100 parts of original casein. The amino-acid mixture was added to the media to give a concentration of 20 mg./100 ml. of L-arginine, L-aspartic acid, L-citrulline, L-cystine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-hydroxyproline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-tryptophan, L-tyrosine and L-valine, and 40 mg./100 ml. of DL-serine and DL-threonine.

When the effects of secretory stimulants were studied, carbamylcholine, pilocarpine or acetylcholine with eserine were either placed in the main compartment of the manometer flask initially or tipped from the side arm after 30 min. incubation. The latter procedure was followed when the effect of secretory stimulants on tissue respiration was studied.

Preparation and incubation of pigeon pancreas. Pigeon pancreas has the advantage over the pancreas of many other species of being relatively homogeneous; it contains very little adipose tissue, and its consistency lends itself well to the slicing technique.

The following procedure was found to give good results and was adopted for the preparation of the tissue. Pigeons were provided with an abundant supply of 'pigeon corn'. About 45 min. to 1 hr. prior to killing 0·09–0·15 mg. of carbamylcholine was administered intramuscularly. Salivation usually began within 5 min., and prostration occasionally occurred. The birds were killed by decapitation, the abdomen was immediately plucked and a substernal incision was made. The duodenal loop, which contains the pancreas, was exteriorized and both lobes of the pancreas were dissected from the duodenum with a pair of sharp scissors. The pancreas and duodenum of pigeons receiving carbamylcholine were usually hyperaemic. Care was taken to avoid puncturing the duodenum in order to prevent contamination of the tissue by the duodenal contents.

The lobes were carefully freed of connective tissue, and slices approximately 0·5 mm. thick were made with the slicer of Stadie & Riggs (1944). The base of the slicer was chilled before use. Unless otherwise specified, the tissue was placed immediately after slicing in a covered crystallizing dish lying in chipped ice. Wetted filter paper was kept in the dish to maintain a high humidity. When sufficient slices had been prepared, they were weighed on a torsion balance and placed in conical manometer flasks (18–26 ml. volume) containing

* Since this paper was submitted it has come to the author's attention that Peters & Anfinsen (1950) have reported a net synthesis of albumin by chicken-liver slices.

the media. Since the amylase activity per mg. dry weight of either lobe of the pancreas agreed within the experimental error (average percentage error between duplicate slices from nineteen pigeons, 5%, extreme range 25%) slices from both lobes could be used, but usually only slices from the large lobe were taken. From 35 to 75 mg. (wet wt.) of tissue were placed in each flask. In each experiment unincubated weighed slices were dried in an oven overnight at approximately 105°, and the ratio of the dry weight to the wet weight (about 0·23) was thus obtained. From this ratio the initial dry weights of the incubated slices were calculated.

The manometer flasks were kept in a tray of ice before they were attached to manometers. From 35 to 45 min. elapsed from the time of killing until all the vessels were placed in the bath. The tissues were incubated for 2 hr. at 40° unless otherwise specified. In some of the experiments gas exchanges were recorded.

Treatment of tissues and media after incubation. Immediately after incubation the vessels were returned to a tray of ice. The slices were removed from the vessels with forceps, drained on Whatman no. 50 filter paper, placed in a small mortar and ground with Calais sand and 2 drops of water for 1·5 min. At the end of this time the mixture had the consistency of a creamy paste. Water (5 ml. minus 2 drops) was then added, and the mixture was ground for another 1·5 min. The mixture was then centrifuged for 15 min. Portions (1 ml.) of the supernatant and the medium were each diluted by adding to 10, 15 or 20 ml. water. The degree of dilution was determined by the expected enzyme activity. The same dilution was used in any one series of experiments. These solutions were either immediately assayed or stored in the refrigerator overnight and assayed on the next day. No change in enzyme activity was observed when the diluted enzyme solutions were stored in the refrigerator for periods up to 5 days.

Methods of assay. Amylase was assayed by the method of Smith & Roe (1949). Since Lintner's soluble starch (Merck) was not initially available, the following modifications of the original procedure were used. A 3% (w/v) solution of Analar soluble starch (obtained from Hopkins and Williams Ltd., 17 St Cross Street, London, E.C. 1) was used instead of the 1·2% (w/v) solution of Lintner's soluble starch (Merck) recommended by Smith & Roe (1949). The use of Analar starch in a higher concentration was made possible because its solutions had a lower viscosity than those of Lintner's soluble starch (Merck). The quantity of I_2-KI solution was four times that recommended by Smith & Roe (1949). The range of the method was increased by the use of higher starch and I_2 concentrations. However, when a second batch of Analar starch was found to differ from the first by a markedly higher viscosity, this brand of starch was abandoned. Lintner's soluble starch (Merck), which had then become available (obtained from Merck & Co., Rahway, N.J., U.S.A.), was used according to the specifications of Smith & Roe (1949).

By assaying several enzyme solutions with the modified method and the method of Smith & Roe (1949), a correction factor (0·87) was obtained to convert the units of the modified method into the units of the method of Smith & Roe (1949).

The approximate maximal amylase activity from each series was determined in a preliminary assay. The time of incubation in the final assay was adjusted accordingly, so that the range of the method was not exceeded. This procedure was permissible because the rate of starch digestion was constant over the maximum period tested (30 min.). The starch-I_2 colours were read in a Beckman spectrophotometer at 620 mμ.

The enzyme activities of the medium and tissue were expressed in units of Smith & Roe (1949) per mg. initial dry weight of tissue. The sum of the medium and tissue amylase activities was referred to as the 'total amylase activity'. It was found that in duplicate experiments the partition of amylase activity between tissue and medium varied more widely than the total amylase activity.

RESULTS

Reliability of amylase assay in slices. When freshly prepared slices of varying weight from different parts of the pancreas were extracted in the same amount of water, the amylase activities found per mg. dry weight agreed within 4% (Table 1). This shows that amylase was completely extracted and was evenly distributed in the pancreas.

Table 1. *Variations in amylase activity of unincubated slices from a single pancreas*

(No carbamylcholine given.)

Dry weight of slice (mg.)	Amylase activity (units of Smith & Roe/mg. dry wt.)
11·0	82
19·8	81
26·2	83
30·2	84

The following substances which were present in the medium of some experiments did not interfere with the amylase assay in the concentrations indicated: carbamylcholine (0·1 mg./100 ml.), acetylcholine (0·1 mg./100 ml.), eserine (0·1 mg./100 ml.), pilocarpine (0·1 mg./100 ml.), supplemented casein hydrolysate (6 mg./100 ml.), bicarbonate saline (1:90 dilution), 2:4-dinitrophenol (10^{-6} M), potassium cyanide (10^{-6} M), sodium iodoacetate (10^{-5} M), serum (1:90 dilution), glucose (2 mg./100 ml.) and the mixture of twenty-one amino-acids (0·2 mg./100 ml. for each amino-acid, except DL-serine and DL-threonine, which were present in 0·4 mg./100 ml.). The concentrations tested were the highest ever present.

Depletion of amylase from the pancreas in vivo. In the beginning of this study the diets of pigeons were not rigidly controlled, nor was carbamylcholine administered prior to killing. Unincubated pancreas slices from sixteen such pigeons gave a mean amylase activity of 160 units/mg. dry wt., range 81–420 units/mg. dry wt. The increase in amylase activity on incubation of such slices was inconsistent. It was thought desirable to use pancreas tissue low in amylase for the study of enzyme synthesis, and attempts were therefore made to deplete the tissue of the enzyme. It is known that the ingestion of food or

cholinergic drugs generally deplete the pancreas of its enzyme stores. Pigeons were therefore provided with a continuous supply of food and were injected with carbamylcholine 45 min. to 1 hr. before killing. The mean amylase level of unincubated pancreas slices from thirteen pigeons was reduced to 53 units/mg. dry wt., range 28–93 units/mg. dry wt. When pancreas slices from these pigeons were incubated under appropriate conditions increases in total amylase activity were consistently observed.

Increase of amylase activity on aerobic incubation. On aerobic incubation in saline containing glucose and supplemented casein hydrolysate the total amylase activity of pancreas slices increased approximately two- to three-fold during 3 hr. of incubation (Table 2). The rate of increase, after an initial lag period during the first 30 min., was fairly constant. Exp. 1 of Table 2 shows that considerable amounts of amylase were discharged into the medium within 30 min. with a concomitant fall in the tissue amylase content. In this experiment the slices were cut dry and kept in the chilled chamber before incubation.

Table 2. *Rate of amylase synthesis by pigeon pancreas slices*

(Bicarbonate saline gassed with 5% CO_2+95% O_2; 0·2% glucose; 0·4% supplemented casein hydrolysate.)

Exp. no.	Period of incubation (min.)	Amylase activity (units of Smith & Roe/mg. initial dry wt.) Medium	Tissue	Total
1	0	0	59	59
	30	16	48	64
	60	21	59	80
	120	31	75	106
	180	31	117	148
2	0	0	52	52
	30	2	55	57
	60	12	70	82
	120	17	131	148
	180	27	153	180

In Exp. 2 of Table 2, in which the slices were suspended in chilled basal saline (similar to the saline of Krebs & Henseleit (1932), but containing 0·004 M-HCO_3^-) before incubation, both the amylase of the medium and tissue rose progressively. The initial rapid rise in the amylase content of the medium and the concomitant fall in the tissue amylase in Exp. 1 were thus probably due to the passive discharge of the enzyme resulting from initial tissue damage rather than to active secretion. The suspension of slices in chilled saline prior to incubation is probably a preferable procedure. Most of the experiments reported in this paper were carried out before this was discovered.

Effect of aerobic and anaerobic incubation on total amylase activity. An increase in total amylase activity upon incubation does not necessarily prove that a complete synthesis of amylase has occurred. An activation of a closely related precursor, analogous to the conversion of trypsinogen to trypsin, might account for such an effect. If this were the case, the increase in total amylase activity might be expected to occur anaerobically, since it is known that the activation of zymogens does not require an external source of energy (Northrop, 1948). The results of aerobic and anaerobic incubation of pancreas slices in saline containing glucose and supplemented casein hydrolysate can be seen in Table 3. There was no increase in total amylase activity when slices were incubated anaerobically, whilst aerobically the total amylase activity rose approximately 100%. These observations support the view that the increase in total amylase activity represents a real synthesis of amylase.

Secretion of amylase. Experiments were carried out to test whether the amylase activity of the medium would be increased in the presence of the secretory stimulant, carbamylcholine. The results are included in Table 3. Aerobically, but not anaerobically, carbamylcholine increased the fraction of amylase in the medium, indicating that the

Table 3. *Synthesis and secretion of amylase under aerobic and anaerobic conditions*

(Bicarbonate saline; 0·2% glucose; 0·4% supplemented casein hydrolysate; phosphorus in centre well when gas phase 5% CO_2+95% N_2.)

Period of incubation (hr.)	Carbamylcholine (mg./100 ml.)	Gas phase	Amylase activity (units of Smith & Roe/mg. initial dry wt.) Medium	Tissue	Total
0	—	—	0	35	35
0	—	—	0	39	39
2	0	5% CO_2+95% N_2	10	17	27
2	0	5% CO_2+95% N_2	11	22	33
2	1	5% CO_2+95% N_2	10	21	31
2	1	5% CO_2+95% N_2	8	28	36
2	0	5% CO_2+95% O_2	19	57	76
2	0	5% CO_2+95% O_2	19	55	74
2	1	5% CO_2+95% O_2	28	41	69
2	1	5% CO_2+95% O_2	30	43	73

Table 4. *Effect of metabolic inhibitors on synthesis and secretion of amylase*

(Bicarbonate saline; 0·2% glucose; 0·4% supplemented casein hydrolysate; phosphorus in centre well when gas phase 5% CO_2+95% N_2.)

Period of incubation (hr.)	Inhibitor added (M concn.)	Gas phase	Amylase activity (units of Smith & Roe/mg. initial dry wt.) Medium	Tissue	Total
0	—	—	0	44	44
0	—	—	0	39	39
2	None	5% CO_2+95% N_2	10	32	42
2	None	5% CO_2+95% N_2	10	29	39
2	2:4-Dinitrophenol (10^{-5})	5% CO_2+95% O_2	17	37	54
2	2:4-Dinitrophenol (10^{-4})	5% CO_2+95% O_2	10	20	30
2	KCN (10^{-5})	5% CO_2+95% O_2	17	42	59
2	KCN (10^{-4})	5% CO_2+95% O_2	10	30	40
2	Sodium iodoacetate (10^{-4})	5% CO_2+95% O_2	14	27	41
2	Sodium iodoacetate (10^{-3})	5% CO_2+95% O_2	20	14	34
2	None	5% CO_2+95% O_2	20	73	93
2	None	5% CO_2+95% O_2	32	68	100

drug had stimulated amylase secretion *in vitro*. Even in the absence of carbamylcholine the amylase activity of the medium was higher in aerobic than in anaerobic experiments, which suggests that 'spontaneous' secretion took place.

Since carbamylcholine had no stimulating effect anaerobically it is probable that the amylase that was present in the medium of anaerobic experiments was due to tissue disintegration rather than secretion.

The 'spontaneous' secretion which occurred aerobically without addition of carbamylcholine may have been due in part to the retention by the pancreas of the carbamylcholine given *in vivo*, but it is noteworthy that it was also observed in experiments in which no carbamylcholine was given prior to killing.

Under the conditions of these experiments the rate of synthesis of amylase was not appreciably affected by carbamylcholine (Table 3). This may have been due to the fact that the glands used in these experiments were largely depleted of their amylase. Under these conditions one would expect the rate of synthesis to be already maximal. It is likely that under some conditions the stimulation of secretion increases the synthesis of enzymes (Langstroth, McRae & Komarov, 1939).

Effects of 2:4-dinitrophenol, cyanide and iodoacetate on synthesis and secretion. Substances which interfere with the generation of usable energy by the cell would be expected to inhibit the synthesis and secretion of amylase. As shown in Table 4, 2:4-dinitrophenol, potassium cyanide and sodium iodoacetate inhibited synthesis over 99% in a concentration of 10^{-4}M. 2:4-Dinitrophenol and potassium cyanide, in a concentration of 10^{-5}M, inhibited synthesis approximately 70%.

Secretion was inhibited 100% by 2:4-dinitrophenol and potassium cyanide in a concentration of 10^{-4}M and approximately 60% by these two drugs in a concentration of 10^{-5}M. Sodium iodoacetate caused marked visible disintegration of the tissue. This, rather than secretion, was probably responsible for the higher medium amylase activities in experiments in which this compound was added (Table 4).

Effect of various media on synthesis of amylase. To study the effects of various media on the synthesis of amylase, slices were incubated in serum and in saline with and without amino-acids (Table 5). The amino-acids were present either as supplemented casein hydrolysate or as the mixture of amino-acids described above. In glucose saline without added amino-acids there was a variable but significant synthesis. Synthesis was greater in serum and greatest in saline containing either supplemented casein hydrolysate or the amino-acid mixture. There was no significant difference between the results using these two sources of amino-acids. Increasing the concentration of supplemented casein hydrolysate from 0·2–0·4% (w/v) had no significant effect on the rate of synthesis. That serum gave lower rates of synthesis than saline media to which amino-acids were added suggests that in serum the amino-acid concentration was the limiting factor. In the saline media, the amino-acid concentration was three to twenty times higher than in serum. The occurrence of some synthesis in saline media to which no amino-acids have been added can be explained by the considerable store of free amino-acids in animal tissues (see Van Slyke & Meyer, 1913).

Table 5 also shows that the substitution of the bicarbonate saline of Krebs & Henseleit (1932) with the modified medium III of Krebs (1950) was without effect on synthesis or secretion. The more physiological concentrations of bicarbonate and carbon dioxide present in the former medium are therefore not essential for synthesis and secretion under the conditions of these experiments.

The comparative effects of various cholinergic drugs on the secretion of amylase. The effects of carbamyl-

Table 5. *Effect of various media on synthesis of amylase*

(0·2% added glucose; for amino-acid mixture see p. 320; in Exp. 1 carbamylcholine tipped into main compartment after 30 min. incubation (final concentration 1 mg./100 ml.).)

Exp. no.	Period of incubation (hr.)	Medium	Gas phase	Amylase activity (units of Smith & Roe/mg. initial dry wt.) Medium	Tissue	Total
1	0	—	—	0	39	39
	0	—	—	0	41	41
	2	Bicarbonate saline	5% CO_2+95% O_2	31	26	57
	2	Bicarbonate saline	5% CO_2+95% O_2	30	25	55
	2	Medium III (Krebs, 1950)	Oxygen	26	28	54
	2	Medium III (Krebs, 1950)	Oxygen	28	26	54
	2	Sheep serum	5% CO_2+95% O_2	24	38	62
	2	Sheep serum	5% CO_2+95% O_2	25	43	68
	2	Bicarbonate saline with 0·2% casein hydrolysate	5% CO_2+95% O_2	32	46	78
	2	Bicarbonate saline with 0·2% casein hydrolysate	5% CO_2+95% O_2	40	44	84
	2	Medium III (Krebs, 1950) with 0·2% casein hydrolysate	Oxygen	39	44	83
	2	Medium III (Krebs, 1950) with 0·2% casein hydrolysate	Oxygen	28	48	76
2	0	—	—	0	31	31
	0	—	—	0	29	29
	2	Bicarbonate saline	5% CO_2+95% O_2	20	22	42
	2	Bicarbonate saline	5% CO_2+95% O_2	20	22	42
	2	Bicarbonate saline with 0·2% casein hydrolysate	5% CO_2+95% O_2	26	32	58
	2	Bicarbonate saline with 0·2% casein hydrolysate	5% CO_2+95% O_2	32	34	66
	2	Bicarbonate saline with 0·4% casein hydrolysate	5% CO_2+95% O_2	31	35	66
	2	Bicarbonate saline with 0·4% casein hydrolysate	5% CO_2+95% O_2	28	37	65
3	0	—	—	0	24	24
	0	—	—	0	32	32
	2	Sheep serum	5% CO_2+95% O_2	14	40	54
	2	Sheep serum	5% CO_2+95% O_2	14	42	56
	2	Bicarbonate saline with 0·4% casein hydrolysate	5% CO_2+95% O_2	16	46	62
	2	Bicarbonate saline with 0·4% casein hydrolysate	5% CO_2+95% O_2	14	51	65
4	0	—	—	0	77	77
	0	—	—	0	69	69
	2	Bicarbonate saline	5% CO_2+95% O_2	36	81	117
	2	Bicarbonate saline	5% CO_2+95% O_2	27	99	126
	2	Bicarbonate saline with amino-acid mixture	5% CO_2+95% O_2	41	127	168
	2	Bicarbonate saline with amino-acid mixture	5% CO_2+95% O_2	32	137	169
	2	Bicarbonate saline with 0·4% casein hydrolysate	5% CO_2+95% O_2	32	121	153
	2	Bicarbonate saline with 0·4% casein hydrolysate	5% CO_2+95% O_2	38	139	177

choline, pilocarpine and acetylcholine with eserine on the secretion of amylase are shown in Table 6.

Weight for weight, carbamylcholine, and acetylcholine with eserine were equally effective in stimulating secretion. Pilocarpine was slightly less effective. Increasing the concentrations of these drugs above 0·1 mg./100 ml. had no greater effect on secretion.

Weight of amylase synthesized. Meyer, Fischer & Bernfeld (1947*a*, *b*) have crystallized amylase from pig pancreas and have assayed its activity by measuring the maltose liberated from starch after digestion with the pure enzyme. Under the conditions of their assay the activity of crystalline amylase was found to be $4{\cdot}0 \times 10^3$ mg. maltose/mg. nitrogen. They found that this preparation of amylase contained 16% nitrogen.

To relate the amylase units of Smith & Roe (1949) to the units of Meyer *et al.* (1947*a*) an extract of pigeon pancreas was assayed by both these methods.

Table 6. *Effect of carbamylcholine, pilocarpine and acetylcholine with eserine on secretion of amylase*

(Bicarbonate saline gassed with 5% CO_2+95% O_2; 0·2% glucose; 0·4% supplemented casein hydrolysate.)

Period of incubation (hr.)	Drug	Concentration (mg./100 ml.)	Amylase activity (units of Smith & Roe/mg. initial dry wt.)		
			Medium	Tissue	Total
0	—	—	0	55	55
0	—	—	0	58	58
2	Carbamylcholine	0·1	41	57	98
2	Carbamylcholine	1·0	34	57	91
2	Carbamylcholine	10	40	53	93
2	Pilocarpine	0·1	27	62	89
2	Pilocarpine	1·0	27	54	81
2	Acetylcholine Eserine	0·1 0·1	38	43	81
2	Acetylcholine Eserine	1·0 1·0	36	47	84
2	Acetylcholine Eserine	10 10	36	51	87
2	None	—	23	58	81
2	None	—	23	52	75

The method of Meyer *et al.* (1947*a*) had to be modified because 'Soluble starch Zulkowski' (Merck) was not available. It was replaced by Lintner's soluble starch (Merck). After the period of starch digestion the tubes were promptly plunged into a boiling-water bath for 5 min. The maltose liberated by the amylase was then measured by the method of Miller & Van Slyke (1936), using a standard maltose solution. One unit of Smith & Roe (1949) was found to be equivalent to 0·15 mg. of maltose liberated by the method of Meyer *et al.* (1947*a*). It follows that an average rate of synthesis (20 amylase units of Smith & Roe/mg. dry wt./hr.) would be approximately 5 μg. amylase/mg. dry wt./hr. This calculation assumes that crystalline amylase prepared by Meyer *et al.* (1947*a*, *b*) is a single enzyme protein. Assuming that α-amylase has a molecular weight of 45,000 (Danielsson, 1947) the rate of amylase synthesis is approximately 10^{-4} μmol./mg. dry wt./hr.

DISCUSSION

Pancreas slices as a tool for studying protein synthesis. The experiments reported here provide evidence that the increase in total amylase activity upon aerobic incubation of pancreas slices is due to a synthesis of amylase and that this increase can be used as a measure of protein synthesis under controlled conditions. It cannot be concluded, however, that the entire amylase molecule is synthesized directly from amino-acids, since it is possible that the enzyme is formed by the addition of a few amino-acids to a polypeptide or protein precursor.

Many workers have recently studied protein synthesis *in vitro* by measuring the incorporation of labelled amino-acids into the proteins of tissue homogenates and slices (Melchior & Tarver, 1947; Winnick, Friedberg & Greenberg, 1947, 1948; Winnick, Moring-Claesson & Greenberg, 1948; Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1949*a*, *b*; Anfinsen, Beloff, Hastings & Solomon, 1947; Frantz, Zamecnik, Reese & Stephenson, 1948; Zamecnik, Frantz, Lotfield & Stephenson, 1948; Rutman, Dempster & Tarver, 1949; Simpson & Tarver, 1950). The phenomenon studied by this method is essentially the 'dynamic state' of tissue proteins. The method measures the rates of exchange of free and protein-bound amino-acids, but gives no information about the net synthesis of protein. Furthermore, the proteins studied are those obtained by precipitation with trichloroacetic acid or similar agents, or by boiling. They thus represent a mixture of tissue proteins. The method presented here gives a measure of a net synthesis of a specific protein.

Enzyme secretion in vitro. The experiments in this paper indicate that pigeon-pancreas slices actively secrete amylase *in vitro*. The *in vitro* secretion of enzymes by digestive glands has been observed previously. Davies, Harper & Mackay (1949), by histological studies, have shown the discharge of secretory granules from isolated cat pancreas incubated in saline media containing pancreozymin or acetylcholine with eserine. In preliminary experiments Edwards & Edwards (1949*a*, *b*) have reported *in vitro* secretion of pepsin by isolated dog stomach. Secretion was apparently increased by acetylcholine, pilocarpine or eserine. Ringer solution was found to be 'rather unsatisfactory' as a nutrient medium (Edwards & Edwards, 1949*b*), and most of the experiments were performed in homologous serum. Edwards & Edwards (1949*a*) did not assay the pepsin of both the medium and tissue to test

whether the 'total peptic activity' (sum of medium and tissue peptic activities) had increased during incubation.

SUMMARY

1. A method is described for measuring the synthesis and secretion of amylase by slices of pigeon pancreas *in vitro*.

2. The amylase content of pancreas tissue can be considerably reduced by abundant feeding of the pigeons and injection of carbamylcholine before killing.

3. An increase in the total amylase activity (sum of medium and tissue amylase activities) occurs when depleted pancreas slices are incubated aerobically in media containing glucose.

4. This increase in total amylase activity requires oxygen and is inhibited by 2:4-dinitrophenol, cyanide and iodoacetate.

5. The increase in total amylase activity is greatest (80–190 %) in saline containing amino-acids, least (40–70 %) in saline without amino-acids and intermediate (60–100 %) in serum. In the presence of amino-acids the increase in total amylase activity corresponds to about 5 μg. amylase/mg. dry wt./hr.

6. In the presence of carbamylcholine, pilocarpine or acetylcholine with eserine, pancreas slices discharge more amylase into the medium than in the absence of these drugs. This discharge of enzyme is dependent on oxygen and is inhibited by 2:4-dinitrophenol and cyanide.

7. The above observations indicate that pigeon pancreas slices synthesize and actively secrete amylase *in vitro*, if respiration is intact.

8. Under the conditions of the experiments synthesis of amylase is not appreciably affected by secretion.

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The Quantitative Recovery and Colorimetric Estimation of Amino-acids Separated by Paper Chromatography

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Numerous methods have been published for the quantitative estimation of amino-acids after separation by the filter-paper chromatography technique of Consden, Gordon & Martin (1944). In all methods the solvent used in the development of the chromatogram is first removed by drying the paper in a current of hot air at a temperature of 70–100° before the estimation of the amino-acid. Many different types of assay have been used, but they can be divided into three groups. In the first group, the intensities of the colours of the spots produced on the chromatograms following treatment with ninhydrin are compared visually (Polson, 1948) or by photometry (Bull, Hahn & Baptist, 1949). For one estimation a large number of chromatograms are required; an accuracy of 10–15 % has been claimed by different workers. The second group includes assays based on the formation of water-soluble amino-acid copper complexes (2 molecules of α-amino-acid to 1 atom copper). The copper complexes have been estimated by polarography (Martin & Mittelmann, 1948) and by determination of the copper colorimetrically (Woiwod, 1949). The latter has obtained results which appear to be reproducible with an accuracy of 5–10 %. The third group includes methods in which the amino-acids are determined colorimetrically after removal from the chromatograms using a ninhydrin reagent.

The ninhydrin method of Moore & Stein (1948) for estimating amino-acids separated by starch column chromatography is very sensitive, accurate and reproducible. In the present work a slightly modified Moore & Stein reagent has been applied to the estimation of amino-acids separated on filter paper. Preliminary experiments showed that this method was more sensitive than the copper complex method of Woiwod (1949), which may be further complicated by the presence in filter paper of a substance which inhibits the completeness of complex formation between copper and some amino-acids (Fowden, 1949). The ninhydrin method of Naftalin (1948) lacked reproducibility, whilst the method of Awapara (1949), in which 1 ml. of a 10 % solution of pyridine and 2 ml. of a 1 % aqueous solution of ninhydrin were added directly to pieces of filter paper containing the amino-acid cut from the chromatograms, was fairly reproducible and sensitive, but was impaired by high and variable 'paper blanks' due to the presence of absorbed ammonia on the paper.

The Moore & Stein reagent gives approximately twice the colour per mol. of amino-acid obtained using the method of Awapara (1949). The amino-acids were estimated whilst still absorbed on pieces of filter paper cut from the chromatograms, thereby eliminating the capillary method of washing the amino-acids from the paper described by Consden, Gordon & Martin (1947), and considerably reducing the manipulation required. After the development of chromatograms in the phenol-ammonia solvent, the concentration of ammonia absorbed on the surface of the filter paper was considerably increased, giving rise to high and variable paper blanks in the colorimetric estimation. The dilute alkali treatment described later to remove this absorbed ammonia, reduced the paper blanks to a low and constant level for equal sized pieces of paper taken from the same sheet. In the determination of 2 or more μg. α-NH_2-N of an amino-acid, the maximum error introduced into the final estimate by the paper blank was then 3 %.

Various procedures were used to remove the solvents from the developed chromatograms, and are described in the Results section. Only the method found to give quantitative recoveries of amino-acids from the chromatograms is given below in the Methods section.

Artificial mixtures of amino-acids were used in the work reported here, but satisfactory results are being obtained with biological materials after desalting by the procedure of Consden *et al.* (1947).

METHODS

Ninhydrin was most conveniently prepared in large batches by the method of Teeters & Shriner (1933), all traces of SeO_2 being removed by additions of small amounts of $SnCl_2$ and repeated recrystallizations from water before use. Hydrindantin was prepared from ninhydrin by treatment with H_2S (Ruhemann, 1911), being recrystallized from acetone. The Moore & Stein ninhydrin reagent was modified to be saturated with hydrindantin, the reduced form of ninhydrin. This change was found to overcome slight interfering effects produced by small amounts of oxidizing agents present in the paper. The results were then found to be more consistent and maximum colour response per mol. of amino-acid was obtained.

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Ninhydrin reagent. Ninhydrin (4 g.) and hydrindantin (80 mg.) were dissolved in 100 ml. of redistilled methyl cellosolve, shown to be free from oxidizing agents by the starch-iodide test. $SnCl_2.2H_2O$ (160 mg.) was then dissolved in 100 ml. of pH 5 citrate buffer, and this solution was added to the first with stirring. After standing for a few hours the precipitated, excess hydrindantin was removed by centrifugation, and the reagent stored under N_2 until required to ensure that its high sensitivity was preserved. All the chemicals used in this reagent were as free from contamination with NH_3 as was reasonably possible, otherwise high reagent blanks resulted in the colorimetric measurements.

Chromatographic technique. The method was essentially that described by Consden *et al.* (1944). Whatman no. 4 filter-paper sheets (56 × 46 cm.) were used in all experiments. The size of the original spots formed in adding the amino-acids to the chromatograms were kept as small as possible, about 6–8 mm. in diameter. The solvents generally used have been phenol saturated with water at the working temperature (a little NH_3 was added to the tank when using this solvent) and the *n*-butanol-acetic acid mixture described by Partridge (1948). All chromatograms were run for about 18 hr. at 25° in a temperature-controlled room. In the development of some of the two-dimensional chromatograms described later, the running time in the *n*-butanol-acetic acid was increased to give a better separation of the amino-acids.

The phenol solvent was removed from the developed chromatograms as follows. The papers were first allowed to dry in cold air for about 1 hr., when the aqueous portion of the solvent evaporated, leaving the papers dry to the touch. The phenol was then removed by washing the papers twice in a bath of redistilled ether, and the chromatograms were finally dried off in cold air. The *n*-butanol-acetic acid solvent was satisfactorily removed by hanging the chromatograms in a current of cold air for 3–4 hr.

In all cases the chromatograms were then heated at 100° for 15 min. The separated amino-acids could then be located as light-blue fluorescent spots on a dark background when the chromatograms were viewed in ultraviolet light. The amino-acids do not fluoresce if this short heating is omitted. Though Gal (1950) has reported losses of amino-acids of up to 7% during prolonged heating in the presence of filter paper, no measurable losses occurred in this 15 min. heating period.

The areas of the chromatogram shown to contain the amino-acids were then removed, together with other pieces of paper of equal size, which served as paper blanks in the colorimetric determinations.

Removal of ammonia. The pieces of paper taken from the dried chromatograms were folded to a convenient size and placed into separate clean 150 × 16 mm. tubes. 0·1 N-NaOH (0·5 ml.) was added to each tube (this volume was just sufficient to wet the paper uniformly) and the tubes placed in a desiccator *in vacuo* overnight over conc. H_2SO_4 (A.R.). Citric acid was then added to bring the papers to approximately pH 5. Alternatively, a calculated extra amount of citric acid was added to the ninhydrin reagent so that pH 5 was obtained after the addition of the reagent to each tube.

If only a few amino-acid determinations were required at any one time, it was found convenient to remove the absorbed NH_3 by the following method, which eliminated the overnight delay but increased the time taken by each determination. After the addition of 0·5 ml. of 0·1 N-NaOH to each tube, they were placed in a boiling-water bath, and a stream of H_2SO_4-washed N_2 was blown through each tube in turn—2 min. was sufficient to remove all the NH_3.

Colour development. Ninhydrin reagent (1·5 ml.) was added to each tube and they were shaken mechanically for 5–10 min. to extract the amino-acids from the paper. The maximum colour production was not obtained on some occasions when the shaking was omitted. The tubes were then placed in a boiling-water bath for 25 min., when colour production was complete. After cooling, 10 ml. of distilled water were added, the tubes shaken and the contents poured into volumetric flasks. Acetone (10 ml.) was added to each tube to complete the extraction of the colour from the papers. The acetone was poured into the appropriate flask and the contents made up to volume, usually 25 ml., with distilled water. In the estimation of small amounts of amino-acid, 1 ml. of reagent was found to be sufficient, and 10 ml. final volumes were used, the volumes of water and acetone used in the colour extraction being correspondingly reduced. The bluish purple colours produced were estimated using a Unicam Spectrophotometer, the percentage light transmission through a 10 mm. tube of solution at $\lambda = 570$ mμ. being measured, and the corresponding optical densities calculated. These optical densities must be corrected for the optical density due to the reagent and paper blanks determined simultaneously on a blank piece of paper of equal area taken from the same chromatogram.

A standard line relating the calculated optical densities with the corresponding known amounts of α-NH_2-N taken can be constructed for each amino-acid, and unknown amounts of amino-acid determined by reference to this standard line.

RESULTS

The lower limit at which the method can be accurately used was found to be dependent upon the certainty with which the spots could be located after development and drying. Under the conditions described, the relative intensities of fluorescence given by 1 μg. α-NH_2-N of each amino-acid on a one-dimensional chromatogram were compared, and the results are presented in Table 1. The table shows that for many amino-acids this amount approached the lower limit needed for accurate location, and in general larger amounts of amino-acids were necessary when two-dimensional chromatograms were used.

When amounts of amino-acids less than 1 μg. α-NH_2-N were used, a parallel location strip was necessary to determine the final positions of the amino-acids. This location strip was treated with ninhydrin and used as a guide in cutting out the amino-acids from the experimental chromatogram. This method of location reduced the lower limit of possible detection by about a factor of 10, but the line of cutting was subject to an appreciable error, since the positions of the amino-acids were only inferred by reference to another chromatogram.

The colours produced from equivalent amounts of the different amino-acids expressed as a percentage of the colour given by leucine are also given in Table 1. The colour response given by all amino-acids was shown to be unaffected by the presence of paper during the colour development stage.

Table 1. *Relative colour responses and intensities of fluorescence given by equimolar amounts of the amino-acids*

Amino-acid	Colour per mol. of amino-acid. (Leucine taken as 100)	Intensity of fluorescence produced from 1 μg. α-NH_2-N of amino-acid
Aspartic acid	96	+
Glutamic acid	108	+
Cystine (0·5 mol.)	49	?
Serine	99	+ +
Glycine	99	+ +
Threonine	93	+ +
Alanine	102	+ + +
Lysine	114	+ + +
Histidine	106	+ +
Arginine	97	+ + +
Methionine	101	+ + +
Leucine	100	+ + +
Isoleucine	102	+ + +
Phenylalanine	90	+ +.
Tyrosine	94	+
Tryptophan	86	?
Valine	106	+ + +
Glutamine	102	+
Proline	32*	–
Hydroxyproline	17*	–

* Colours read at $\lambda = 440$ mμ. and compared with leucine read at $\lambda = 570$ mμ.

+ + +, Strong; + +, moderate; +, weak; ?, doubtful; –, absent.

A convenient working range when using 25 ml. final volumes and 10 mm. tubes was found to be from 1 to 10 μg. α-NH_2-N of an amino-acid. The plot of the calculated optical densities against the corresponding concentrations of the amino-acids were linear over this range. Fig. 1 illustrates this point using alanine. With the solutions suitably diluted for use with the spectrophotometer, the linearity of the plot could be extended to 30 μg. α-NH_2-N; this was often an upper limit to the amount of an amino-acid that could be separated from a mixture with good resolution on a paper chromatogram.

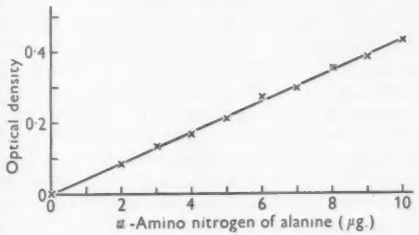

Fig. 1. Optical densities read at $\lambda = 570$ mμ. for increasing amounts of alanine.

The recoveries of standard amounts of amino-acids after flowing on chromatograms were investigated using the standard method of removing the developing solvent by heat. A mixture of aspartic acid, glutamic acid, glycine, threonine, alanine, histidine and phenylalanine, was applied to paper strips (50 μl. spots containing 8 μg. α-NH_2-N of each amino-acid) and chromatographed in phenol. After drying in hot air at 80° for 1 hr. the spots of the separated amino-acids were located using ultraviolet light, and cut out from the papers, together with suitable paper blanks. The recoveries of the amino-acids in all the experiments were based on a comparison of the optical density given by the recovered amino-acid to that given by the standard amount of the same amino-acid when added to a paper blank. The mean recovery values are given in Table 2. In general, the individual results were within 5 % of the mean value.

Table 2 also shows the mean recovery values of the same amino-acids after two-dimensional chromatography. These recoveries were found to be lower than those obtained in one-dimensional chromatography; the scatter of individual results about the mean value was again not greater than 5 %.

Table 2. *Recoveries of some amino-acids taken from developed chromatograms dried at* 80° *for* 1 *hr.*

Amino-acid	Mean percentage recovery after one-way phenol chromatography (four results)	Mean percentage recovery after two-dimensional chromatography (four results)
Aspartic acid	87	80
Glutamic acid	88	72
Glycine	86	76
Alanine	85	71
Threonine	86	73
Phenylalanine	80	60
Histidine	70	49

Using a typical amino-acid, alanine, the percentage recovery was shown to be independent of the amount of amino-acid used, and the distance flowed by the amino-acid on one-dimensional chromatograms. Tables 3 and 4 give the results of these experiments.

The remaining experiments were designed to investigate the nature of the losses encountered during chromatography and to provide a satisfactory method for their elimination, thereby reducing the labour needed for each determination. The apparent loss of alanine during the chromatography process averaged about 8 % when *n*-butanol-acetic acid was the solvent and about 20 % when phenol was used. The constancy of the recovery values obtained for each solvent suggests that the loss could not have been due to trailing of the amino-acid down the paper, for this would have entailed greater losses as the distance of the amino-acid flow increased. Decomposition of amino-acid in the presence of solvent

during the development of the chromatogram was equally unlikely since the amount of decomposition would have been expected to increase, giving a decreased recovery when longer times of running were used.

Table 3. *Recoveries of different concentrations of alanine taken from chromatograms dried at* 90–100° *for* 1 *hr.*

Alanine chromatographed (μg.)	Mean percentage recovery of alanine using *n*-butanol-acetic acid as solvent (four results)	Mean percentage recovery of alanine using phenol as solvent (four results)
10	78	78
20	92	78
40	91	82
50	90	86
60	92	78
70	92	87

Table 4. *Recovery of alanine after flowing for different distances on chromatograms finally dried at* 90–100° *for* 1 *hr.*

Distance travelled by alanine spot (cm.)	Mean percentage recovery of alanine using *n*-butanol-acetic acid as solvent (four results)	Mean percentage recovery of alanine using phenol as solvent (four results)
3	95	.
6	95	75
10	95	81
16	95	77
22	91	.
28	94	77

Losses of amino-acid due to the formation of complexes, having different R_F values, with copper present as an impurity in the filter paper were shown not to occur by comparing the mean recovery of 8 μg. α-NH_2-N of alanine run in phenol to that of alanine run in phenol to which 0·1 % α-benzoin oxime (cupron) was added. The presence of cupron in the solvent would greatly reduce any complex formation between the alanine and copper. The mean values of the percentage recoveries of alanine found for the two solvents were not significantly different ($P > 0{\cdot}05$ for 't' test on difference of means of 76·2 and 79·2 % respectively).

In all the experiments described previously, the paper chromatograms were dried in a hot-air oven at temperatures from 80 to 100° for about an hour. Evidence had accumulated suggesting that the losses were greater when either the drying temperature or time was increased. Using amounts of alanine containing 8 μg. α-NH_2-N the recovery from a chromatogram run in phenol and dried at 80° was shown to fall from 82 % after drying for 1 hr. to 73 % after drying for 3 hr.; again 90 % of the alanine was recovered from a phenol chromatogram dried at a temperature of below 50° for 6 hr. whilst only 69 % was recovered after drying at 100° for 1 hr. Standard amounts of alanine were added to 'blank' areas of the paper chromatograms whilst still saturated with solvent after developing in phenol, and after drying, losses of the added alanine were identical with those found for the alanine which had been run on the chromatogram. When alanine was added to the untreated dry Whatman no. 4 paper, and then subjected to the heat treatment used to dry the chromatogram, no loss occurred.

The losses of amino-acid occurring during chromatography appeared to be due to the partial decomposition of the amino-acids in the presence of solvents at the high drying temperatures used, giving compounds no longer reactive towards ninhydrin, or whose colour response per mol. was much less than that given by the original amino-acids. Drying for longer periods at temperatures below 50° reduced the losses of amino-acid to about 10 %, and for many types of experiment this procedure could be used to obtain rough values in amino-acid assays.

In an attempt to overcome such heat losses Fowden & Penney (1950) suggested the use of ether washing to remove phenol from developed chromatograms, and showed that alanine and phenylalanine could be quantitatively recovered from one- and two-dimensional chromatograms after this treatment. The scope of this earlier work has now been widened to include most of the naturally occurring α-amino-acids.

The recovery values of the amino-acids after flowing on one-dimensional phenol chromatograms are given in Table 5. The recoveries obtained when ether washing was used to dry the papers are compared with those obtained after drying at 80° for 3 hr. Strict comparison was made possible by taking a separate sheet of paper for each amino-acid, and treating in the following manner. Six spots, each containing 8 μg. α-NH_2-N of the amino-acid under investigation, were applied to the top edge of the paper. After developing, each sheet was cut along the direction of flow whilst still saturated with solvent, giving two halves each containing three spots. One half of the paper was then ether-washed and the other heat-dried.

The recoveries of lysine and histidine tended to vary; this was attributed to the tailing that these two amino-acids show in phenol solvent systems. The losses occurring in the hot-air drying of chromatograms varied considerably for the different amino-acids, but as seen from Table 5 most of the amino-acids could be quantitatively recovered from one-way phenol chromatograms provided they were dried by ether washing. The losses of amino-acid produced in the hot-air drying of chromatograms run in *n*-butanol-acetic acid were found to be of the order of 10 %; when these chromatograms were

Table 5. *Comparison of the recoveries of the amino-acids* (8 μg. α-NH_2-N) *from phenol run chromatograms after drying at* 80° *for* 3 *hr. and at laboratory temperature by ether washing*

Amino-acid	Mean percentage recovery using ether washing (three results)	Standard deviation from the mean value (three results)	Mean percentage recovery using hot-air drying (three results)	Standard deviation from the mean value (three results)
Alanine	99	3·7	74	0·9
Phenylalanine	101	0·8	71	2·9
Leucine	97	0·8	66	3·9
Isoleucine	98	1·1	61	2·5
Glycine	98	4·2	70	3·8
Glutamic acid	100	2·5	74	3·4
Aspartic acid	98	1·5	82	3·2
Serine	96	1·8	77	4·8
Threonine	95	3·5	76	1·7
Arginine	98	0·9	73	2·3
Lysine	Variable 85–100	.	About 65	.
Histidine	About 80	.	About 60	.
Glutamine	About 70	.	About 48	.
Methionine	91	2·1	87	1·3
Valine	101	2·6	72	1·4
Tryptophan	89*	1·9	67	3·1
Cysteic acid	86*	2·4	76	4·1

* These amino-acids were located using 0·02% ninhydrin solution, before estimation.

dried in a stream of cold air no losses were encountered.

Table 6 gives the results of similar experiments using 2 μg. α-NH_2-N samples of several amino-acids. There is still quantitative recovery at this lower concentration. The standard deviations from the means (four results) rarely exceeded 5%.

Table 6. *Recovery values of amino-acids* (2 μg. α-NH_2-N) *taken from phenol chromatograms dried by ether washing*

Amino-acid	Mean percentage recovery after drying by ether washing (four results)	Standard deviation from the mean value (four results)
Alanine	95	2·1
Leucine	97	5·9
Glutamic acid	94	3·8
Aspartic acid	92	3·2
Serine	95	4·1
Arginine	100	4·5
Valine	102	1·0

An attempt was made to extend the range of estimation down to 0·5 μg. α-NH_2-N amounts of amino-acid, but at these concentrations the positions of the spots after chromatography had to be found using a location strip, and the recovery values obtained were far more variable. The mean value obtained was therefore subject to an appreciable error.

The ether-washing method was used to dry two-dimensional chromatograms after the separation of ten amino-acids contained in a synthetic mixture; the recovery values obtained for each amino-acid are given in Table 7. The recoveries given are the mean of eight separate experiments.

Table 7. *Recoveries of amino-acids* (8 μg. α-NH_2-N) *from two-dimensional chromatograms dried at laboratory temperature by ether washing*

Amino-acid	Mean percentage recovery after drying by ether washing (eight results)	Standard deviation from the mean value (eight results)
Arginine	100	5·6
Leucine	96	3·8
Valine	96	3·4
Phenylalanine	93	4·5
Alanine	99	2·0
Threonine	100	2·4
Glycine	102	7·6
Serine	102	6·6
Glutamic acid	92	2·2
Aspartic acid	95	3·8

The recoveries of the amino-acids obtained in the experiments described show that any losses of amino-acid occurring during the development of the chromatograms must be extremely small, and for practical purposes may safely be ignored.

When the improvements described for the drying and colour development stages and in the removal of absorbed ammonia are carefully followed, the result of a single assay of an amino-acid in a mixture may be expected to have an error of not more than 5% of the true value.

DISCUSSION

The results obtained in this investigation indicate that the nature of the losses previously encountered in the paper-chromatographic separation of amino-acids may depend largely upon the final method of

estimation used. Results obtained, using the ninhydrin method described here, failed to show any increased loss after longer times of running as found by Woiwod (1949) using a copper complex method of estimation. It is inconceivable that this fundamental difference is due to slight differences in the conditions under which the chromatograms were developed, and a possible explanation for these progressive losses when using the copper method of assay is the presence in paper of a substance which inhibits complex formation (Fowden, 1949). On the other hand, hot-air drying seems to produce much smaller losses when the amino-acids are estimated by the copper method than when the ninhydrin method is used in their estimation. The nature of the change taking place during the hot-air drying is still obscure, but it is conceivable that, though the product formed is incapable of reacting with the ninhydrin reagent, it may still be capable of forming a complex with copper. The author has been unable to demonstrate any loss of ammonia by amino-acids when dried on filter paper in the presence of solvents under the conditions usually used in chromatographic practice. The method of amino-acid assay described in this paper has eliminated all serious losses, and whilst some means of eliminating the apparent losses in the copper method of determination is still being investigated, it seems that the more sensitive ninhydrin method will be more useful for future work.

The labour involved in the determination of amino-acids in mixtures using the ninhydrin method is considerably less than in the copper method, since the preparation and running of control chromatograms is unnecessary. If results of the highest degree of accuracy are not required, a single amino-acid may be used as the standard in the colorimetric determination of several amino-acids in an unknown mixture, for the relative colour responses per mol. of the different amino-acids have been compared and found to be reproducible. The number of separate determinations necessary can therefore be further reduced.

No attempt was made to estimate proline or hydroxyproline in this work. Two serious difficulties were found in the determination of these amino-acids by the methods described. The colours given by the amino-acids with the ninhydrin reagent were yellow or yellow-brown, and showed a maximum light absorption at a wavelength $\lambda = 440$ mμ. Even when the colours produced were determined using this wavelength and compared with those given by the other amino-acids measured at $\lambda = 570$ mμ., the colour response per mol. of proline was less than one-third and of hydroxyproline about one-sixth of that given by leucine. Such a loss of sensitivity in the method of determination causes a corresponding increase in the errors likely to arise in the colorimetric estimation. All methods employing ninhydrin suffer from this defect with proline and hydroxyproline, but since these amino-acids form the normal type of complex with copper (2 mol. of amino-acid with 1 atom of copper), they behave in a manner identical with all the other α-amino-acids in the copper method of determination. It was also found necessary to use much larger amounts of proline and hydroxyproline before these amino-acids were visible as fluorescent spots when viewed in ultraviolet light. These two differences from the general behaviour of the α-amino-acids seriously limit the usefulness of the ninhydrin method for the determination of micro-amounts of proline and hydroxyproline.

SUMMARY

1. A ninhydrin method for the estimation of amino-acids after separation by filter-paper chromatography has been described. The applications to synthetic mixtures of pure amino-acids are given. The method uses the Moore & Stein (1948) ninhydrin reagent in a slightly modified form, and can be used in the determination of 1–30 μg. α-NH_2-N of an amino-acid.

2. Ammonia absorbed on the paper has been shown to be responsible for the high and variable values of the paper blank previously obtained in ninhydrin colorimetric methods and a means of easily removing this ammonia is described. The residual paper blank after this treatment is found to be low and constant in value for pieces of paper of equal size.

3. When chromatograms were dried in hot air at 70–100°, 20–30 % losses of α-amino-nitrogen were found for many of the amino-acids determined by the method described. When the chromatograms were dried at room temperature after removal of the solvent by washing with ether, the losses were completely eliminated and quantitative recoveries of the amino-acids made possible. This new method of drying the papers has made control chromatograms of standard amounts of amino-acids unnecessary, and greatly reduced the labour involved in amino-acid determinations.

4. No evidence of losses of amino-acid arising during the running of chromatograms due to trailing or heavy metal complex formation was found.

I wish to express my thanks to Prof. B. S. Platt for his interest in this work.

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The Enzymic Oxidation of Pyridoxal by Liver Aldehyde Oxidase

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Huff & Perlzweig (1944) have shown that pyridoxic acid (3-hydroxy-5-methoxy-2-methylpyridine-4-carboxylic acid) is normally present in human urine, and that the amount excreted may be increased by the intake of vitamin B_6, usually administered in the form of pyridoxine. As pyridoxal phosphate is known to be active as a coenzyme of amino-acid decarboxylases, it was reasonable to assume that the acid might be produced in the body by a direct oxidation of the aldehyde. In this paper we have been able to show that pyridoxal can, in fact, be oxidized in the presence of liver aldehyde oxidase and that the reaction product is pyridoxic acid. The course of the reaction has been followed by measuring the rate of disappearance of the aldehyde and the accompanying increase in fluorescence, and the reaction product has been isolated and identified as pyridoxic acid.

EXPERIMENTAL

Most of the enzymic studies were carried out using aldehyde oxidase (Knox, 1946) prepared from rabbit liver according to the method of Gordon, Green & Subramanyan (1940). This method was, however, far from satisfactory as the potency and stability of different preparations varied unaccountably. The enzyme seldom remained active for longer than 1 week and frequently lost its activity within a few hours after preparation. Moreover, on numerous occasions, the final $(NH_4)_2SO_4$ precipitate contained a heavy white sediment which could not be redissolved and appeared to adsorb the enzyme irreversibly, so that it was impossible to obtain an active preparation. Although it is not possible to state a definite reason, it appears that in general this occurred when the liver used was unusually heavy and light in colour. On such occasions all the soluble fractions were very turbid, and the final $(NH_4)_2SO_4$ precipitate was dense and almost colourless. Activity measurements at the different stages of the purification process have shown that in these cases the enzyme was originally present in the liver homogenate and was carried down with the $(NH_4)_2SO_4$ precipitate. One suggestion is that the difficulty in separating the enzyme might be due to the presence of large quantities of glycogen.

Because of these difficulties we have, in addition, used a preparation of aldehyde oxidase from horse liver for the isolation of the end product. This enzyme, although less fractionated than the rabbit-liver preparation, had retained its potency during 4 months' storage in the refrigerator, but the high concentration of proteins in the preparation rendered it unsuitable for reaction studies in the fluorimeter.

Crystalline pyridoxal hydrochloride (Merck) was used for the substrate solutions. A stock solution containing 10 mg./ml. remained stable for about 1 month when kept in the refrigerator without exposure to light. Fresh dilutions of this solution were prepared for each test, and all tests were carried out in tubes wrapped in tin foil to minimize the effect of light. The enzymic oxidation of pyridoxal was demonstrated by the use of three independent methods.

Decolorization of methylene blue. This method was used only in preliminary tests to show that pyridoxal undergoes oxidation in the presence of aldehyde oxidase. Determinations were carried out in evacuated Thunberg tubes which contained the substrate in 0·1 M-phosphate buffer, pH 7, in the main tube and the enzyme in the side arm. After mixing, the oxidation was followed by measuring the decolorization of the methylene blue through the decrease in absorption at 660 mμ., using the Coleman Junior spectrophotometer. No decolorization was observed in blank tubes containing no enzyme. In this way pyridoxal was shown to be oxidized in the presence of the two liver preparations mentioned. Xanthine oxidase, however, appeared to be inactive towards pyridoxal.

* From the Postgraduate Medical School, University of London, on a scholarship from the Danish Government to British Postgraduate Students.

Determination of the aldehyde concentration during the course of the oxidation. The aldehyde concentration can be simply determined by addition of alkali to the 2:4-dinitrophenylhydrazone. A pink colour, proportional in depth to the amount present, is then produced (Kalckar, Kjeldgaard & Klenow, 1950). In the case of pyridoxal, the resulting colour is unstable, so that it was necessary to take readings at carefully timed intervals after addition of the alkali. This factor reduces the accuracy of the method, but it was possible to obtain fairly good, comparative results.

To follow the course of an oxidation, samples containing up to 10 μg. of pyridoxal were taken from an enzyme incubation mixture and added to 0·2 ml. of 0·5% 2:4-dinitrophenylhydrazine in 0·1 N-HCl. Water was added to bring the volume up to 1 ml., and the solution was heated to boiling. After cooling, 3 ml. of 2 N-NaOH were added, and the colour was read exactly 30 sec. later in the Coleman spectrophotometer at 500 mμ. A blank sample and a standard containing 10 μg. of pyridoxal hydrochloride were included in each set of determinations.

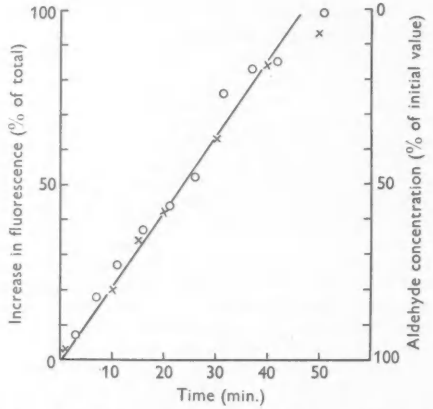

Fig. 1. Relation between the increase in fluorescence and the decrease in aldehyde concentration during oxidation. Pyridoxal hydrochloride (1 mg./ml.) (0·2 ml.) was incubated with 0·8 ml. of rabbit-liver aldehyde oxidase and 3 ml. of 0·1 M-phosphate buffer pH 7. For aldehyde determinations 0·2 ml. samples were pipetted into 0·2 ml. of 0·5% 2:4-dinitrophenylhydrazine. The colour was developed as described in the text. Water was added to both standard and blank so that each tube contained 4 ml. For fluorescence determinations 0·1 ml. samples were pipetted into 1 ml. of phosphate, pH 7, and readings were taken immediately. ○, fluorescence; ×, aldehyde concentration.

As the method was neither sensitive nor accurate enough to be of use in the detailed study of the oxidation, its chief value lay in establishing the validity of using the increase in fluorescence as a measure of the reaction. By measuring both the aldehyde concentration and the fluorescence during the course of a reaction, it was possible to show that the decrease in aldehyde concentration was accompanied by a parallel increase in fluorescence. This relationship is illustrated in Fig. 1.

Fluorescence measurements. Although the fluorescence of pyridoxic acid is almost at its lowest at pH 7, it is still far stronger than that of pyridoxal at the same pH, so that the total increase during a reaction is about tenfold. Measuring this increase is therefore a sensitive and easy method of following the course of an oxidation by continuous observations.

Measurements were carried out in a Farrand multiplier fluorimeter which was set to the required sensitivity with a standard solution of quinine sulphate (0·03 μg./ml.). An appropriate volume of enzyme was added to the substrate contained in 0·1 M-phosphate buffer pH 7, and the fluorescence was recorded at timed intervals. The most convenient substrate concentration was 5–15 μg. of pyridoxal hydrochloride/ml., and, in general, an attempt was made to use an amount of rabbit liver aldehyde oxidase which would complete the reaction within 30 min. when acting on 10 μg. of substrate. Under such conditions the volume of enzyme used was generally 10–50 μl., depending on the potency of the preparation. A blank containing no enzyme never showed any considerable change in fluorescence at pH 7.

RESULTS

By means of fluorimetric measurements the pH optimum of the reaction was established. Since the fluorescence of the resulting acid varies greatly with pH it was necessary to allow the reaction to go to completion, and the rate of reaction during the first 5 min. was expressed as a percentage of the total increase in fluorescence. As in the case of other aldehydes which are oxidized by liver aldehyde oxidase, the optimum pH lies near 7 (Fig. 2).

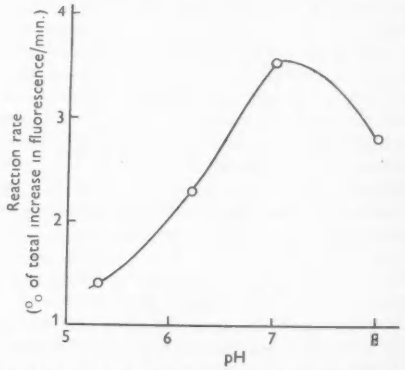

Fig. 2. Rate of oxidation of pyridoxal by liver aldehyde oxidase at different pH values as measured by the rate of increase in fluorescence, which was followed after 0·05 ml. of rabbit-liver aldehyde oxidase had been added to 5 μg. of pyridoxal hydrochloride in 1 ml. of 0·1 M-phosphate buffer of various pH values. The reaction was allowed to go to completion, and the reaction rate is expressed as percentage of the total increase per min. during the initial 5 min. of reaction. In this, as also in other experiments, the standard was arbitrarily set to a fluorescence value of 50 units.

Inhibition by antabuse

It has been shown that antabuse (tetraethylthiuram disulphide) (Hald & Jacobsen, 1948) inhibits the oxidation of acetaldehyde and other aldehydes by liver aldehyde oxidase (Kjeldgaard, 1949). The same effect has now been demonstrated in the oxidation of pyridoxal, and the influence of increasing concentrations of antabuse on the rate of oxidation is illustrated in Fig. 3.

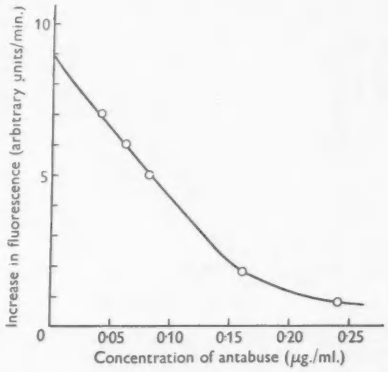

Fig. 3. The effect of varying concentrations of antabuse on the rate of oxidation of pyridoxal. To tubes containing 0·01 mg. of pyridoxal hydrochloride in 1 ml. of 0·1 M-phosphate buffer, pH 7, were added different volumes of an ethanolic solution of antabuse (16 μg./ml.). Each reaction was carried out with 0·02 ml. of rabbit-liver aldehyde oxidase and followed fluorimetrically. The rate of reaction is expressed as the increase in fluorescence per min. during the first 5 min.

The inhibitory effect of antabuse may be reduced by increasing the substrate concentration (Fig. 4). If the rate of oxidation of pyridoxal is plotted against the substrate concentration a curve such as is normal for the majority of enzyme reactions is produced. There is good agreement with the Michaelis-Menten equation over the range recorded, and the Michaelis constant under the conditions of the experiment was found to be $6{\cdot}3 \times 10^{-7}$ M. In the presence of a constant concentration of antabuse the velocity-substrate concentration curve has a similar shape but less slope (curve *B*), so that the Michaelis constant at a concentration of 0·125 μg. of antabuse/ml. had increased to a value of $4{\cdot}1 \times 10^{-6}$ M. When the reciprocal velocity was plotted against reciprocal substrate concentration two straight lines were obtained which met on the *y*-axis (Fig. 5), showing that the velocity at infinite substrate concentration is unaffected by the presence of antabuse. From this it may be concluded that the inhibition by antabuse is of a competitive nature.

Isolation and identification of the end product

Preliminary tests carried out on the product of a reaction whose course to completion had been followed fluorimetrically showed that: (1) The

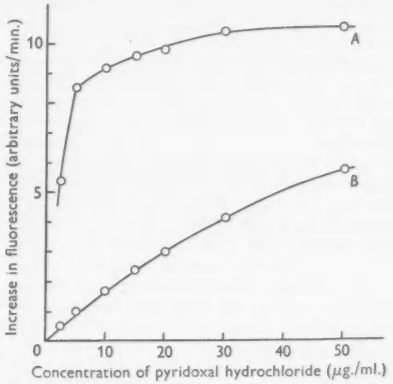

Fig. 4. Effect of substrate concentration on the oxidation of pyridoxal in absence and presence of antabuse. Different volumes of pyridoxal hydrochloride (0·1 mg./ml.) were added to 0·1 M-phosphate buffer, pH 7 + 0·005 ml. of ethanol to give a final volume of 1 ml. The reaction was carried out with 0·015 ml. of rabbit-liver aldehyde oxidase (curve *A*). For curve *B* the reactions were carried out in the same way, but to each tube was added 0·005 ml. of a solution of antabuse in ethanol to give a final concentration of 0·125 μg./ml.

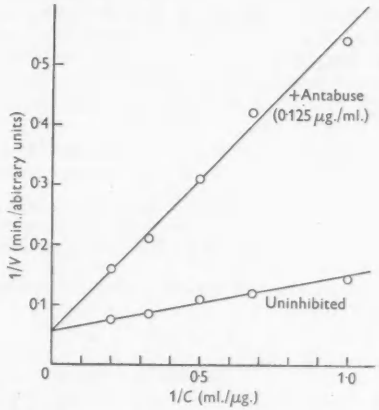

Fig. 5. To demonstrate the competitive nature of the antabuse inhibition. The experimental conditions were the same as for fig. 4: *V*, reaction velocity; *C*, pyridoxal hydrochloride concentration.

product was soluble in butanol and insoluble in ether. (2) The fluorescence varied with pH, with a maximum value at pH 3–4. (3) In 0·5 N-hydro-

chloric acid, after heating in a boiling-water bath for 30 min. the fluorescence greatly increased and the maximum value now occurred at pH 9. As all these properties coincided with those mentioned by Huff & Perlzweig (1944) in the description of pyridoxic acid, we proceeded to isolate the end product by the method described by these authors.

Pyridoxal hydrochloride (100 mg.) was dissolved in 10 ml. of 0·1 M-phosphate buffer pH 7, and incubated with 2 ml. of horse-liver enzyme at 34°. After 24 hr. the reaction was considered to have reached completion as there was no detectable aldehyde present and the fluorescence had reached a steady value. To remove the enzyme the mixture was saturated with Na_2SO_4 and heated to boiling. After filtering and washing the residue with hot water, the clear, yellow solution, which contained about 86 mg. of the acid as indicated by fluorescence measurements, was evaporated to dryness in a current of air. The yellow residue was extracted five times with 10 ml. portions of hot ethanol, and the extract was again evaporated to dryness. The extractions were repeated twice with progressively smaller volumes of ethanol. The final residue was dissolved in 3 ml. of hot 0·1 N-NaOH. After cooling, a few drops of 5 N-HCl were added, when small white crystals began to appear. These were filtered off after standing in the refrigerator overnight, washed rapidly with distilled water, and twice recrystallized from pure pyridine.

The crystals resembled small needles; they were slightly soluble in water, easily soluble in alkali, and insoluble in acid except on prolonged heating when conversion to the far more soluble lactone took place, as indicated by an increased fluorescence. The melting point of the acid was around 245–248° (uncorr., decomp.). The mixed melting point with pyridoxic acid prepared by Huff & Perlzweig was identical. Further comparisons were made, establishing the pH-fluorescence relationships of the two acids and their lactones (Table 1). The ultraviolet absorption spectra of both the acids and their lactones were also found to be identical, although the absolute maximum absorption of the enzymic oxidation product was somewhat lower than that of the synthetic acid. Fig. 6 shows the absorption spectrum of pyridoxal together with those of enzymically formed pyridoxic acid and its lactone, all at pH 7·16.

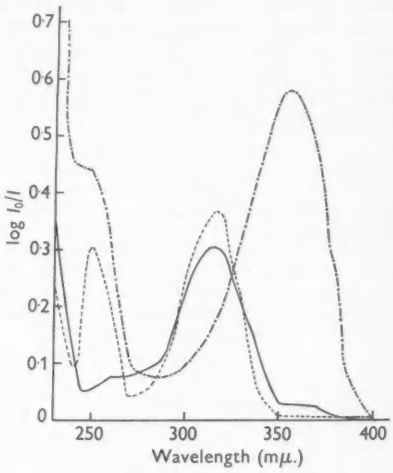

Fig. 6. Absorption spectra of pyridoxal and of enzymically formed pyridoxic acid and its lactone. The solutions contained 10 μg./ml. of pyridoxal hydrochloride, pyridoxic acid and the lactone (prepared as previously described) in 0·2 M-phosphate, pH 7·16. The ultraviolet absorption was measured in the Beckman spectrophotometer. ——, pyridoxic acid; - - - - -, pyridoxal hydrochloride; –·–·–·, pyridoxic acid lactone.

Table 1. *Comparison of synthetic and enzymically produced pyridoxic acids and their lactones by determination of the fluorescence at various* pH *values*

(Pyridoxic acid (1·11 mg.) and the enzymic reaction product (1·26 mg.) were dissolved in 10 ml. of water. Of this solution 0·01 ml. was added to 1 ml. of buffer for readings in the fluorimeter. For lactone formation 2 ml. of the above acid solution were heated with 0·5 ml. of 2 N-HCl for 30 min., diluted to 10 ml. and 0·01 ml. of the final solution was added to 1 ml. buffer as before.

Buffer solutions: pH 1, 0·1 N-HCl; pH 2–8, McIlwaine standard buffer; pH 9, 0·1 M-sodium pyrophosphate.)

	Acid			Lactone		
pH	Synthetic	Enzymic	Standard setting	Synthetic	Enzymic	Standard setting
1	29	33	25	—	—	—
2	45	50	25	—	—	—
3	44·5	47	25	10	10	50
4	41	45	25	11	11	50
5	23	26	25	18	20	50
6	15	16	25	49·5	54	50
7	11	13	25	74	83	50
8	11	12	25	62 (124)	64 (128)	25
9	—	—	—	74 (370)	88 (440)	10

Figures in brackets are calculated to give the values as if the standard setting had been at 50 throughout.

SUMMARY

1. Pyridoxal can be oxidized by liver aldehyde oxidase.

2. The oxidation is competitively inhibited by antabuse.

3. The end product of the reaction has been isolated and identified as pyridoxic acid.

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The Amino-Acid Composition of Two Yeasts used to Produce Massive Dietetic Liver Necrosis in Rats

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Although yeast consists mainly of protein and is easy and cheap to produce in bulk, it is not a good source of dietary protein. For example, Hock & Fink (1943*a*, *b*) showed that when low-protein diets containing different types of yeasts as the main source of protein were fed to young rats, the animals failed to grow normally and after some time died from acute liver lesions consisting of extensive necrosis and haemorrhages. The yeasts varied both in their growth-promoting properties and in their tendency to produce liver lesions (necrogenic properties); there appeared to be some relation between the sulphur/nitrogen ratio of the yeasts and their necrogenic properties, the more necrogenic diets having lower proportions of sulphur. Liver necrosis in rats has also been produced by feeding diets containing suboptimal amounts of other proteins, such as casein or soya-bean meal, as the main source of protein. The lesions appear to be caused by a condition similar to that occurring in rats fed the yeast diets, since development of necrosis on all these diets could be prevented by extra cystine or methionine (Weichselbaum, 1935; Hock & Fink, 1943*a*; Matet, Matet & Fridenson, 1947; Himsworth & Glynn, 1944*b*; György & Goldblatt, 1949).

Necrosis has been produced in this laboratory by using low-protein diets containing baker's yeast as the sole source of protein (Himsworth & Glynn, 1944*a*). Similar diets containing brewer's yeast have been shown by György to be considerably less necrogenic (György, 1948; György & Goldblatt, 1949). Since it seemed probable that the difference in types of yeast used in the diets accounted for the different results obtained in the two laboratories, samples of yeast were exchanged in order to compare their chemical composition and their nutritional properties for the same strain of rat.

The chemical investigation was confined to the amino-acid fractions, particularly sulphur-containing amino-acids. Tocopherol deficiency is another factor involved in the production of necrosis (Schwarz, 1944; Himsworth & Lindan, 1949), but since tocopherol is known to be absent from all types of yeasts (György, 1948, private communication) it could not have accounted for the differences observed. This paper describes the results of investigations on the nitrogen, sulphur and amino-acid contents of our baker's yeast and György's brewer's yeast. Because of the possibility that the extractable and therefore immediately available amino-acids and peptides might be of importance in the nutritional properties of the yeasts, separate investigations were made on the amino-acids present in the cell residues insoluble in 75% ethanol and on

the extractable amino-acids and peptides. The comparison of the nutritional properties of the two yeasts is reported in the following paper (Lindan & Work, 1951).

EXPERIMENTAL

Materials. The following yeasts were analysed: dried baker's yeast (DCL no. *GB* 161) marketed by Distiller's Co. Ltd. London, and dried brewer's yeast (Fleischmann's type 50*B*) marketed by Standard Brands Inc., U.S.A., provided by Dr P. György. All our analyses were carried out on the same bulk samples of each yeast.

Elementary analysis. N was estimated by the standard micro-Kjeldahl method. Total S estimations were carried out after digestion with HNO_3 and $HClO_4$ according to Masters (1939); sulphate S was determined on material hydrolysed for 10 hr. with 6N-HCl. All estimations were carried out in duplicate on 2 g. samples, to avoid sampling errors due to inhomogeneity of material.

Amino-acid analysis. Most of the amino-acid assays, with the exception of cystine and methionine, were carried out by paper chromatography of yeast fractions prepared as follows: the yeast, finely powdered by grinding and sieving if necessary, was extracted with 75% (v/v) ethanol (10 ml./g. of dried yeast) for 24 hr. with shaking in a centrifuge tube. The ethanol was removed by centrifugation and replaced by an equal volume of fresh solvent; the process was repeated three times. The progress of extraction was followed by estimation of total N in the separate extracts.

Since the ethanol extracts could not be used directly for paper chromatography owing to interference from extracted substances other than amino-acids, the method of Awapara (1948) for preparing tissue extracts was used. The ethanol extracts were combined and shaken with 3 vol. of $CHCl_3$. The clear aqueous supernatant phase, which separated from the bulky ethanol-$CHCl_3$ phase on standing or after centrifugation, was concentrated *in vacuo* to a total N concentration of 2·5 mg./ml. This solution, which contained the bulk of the extractable amino-acids of the yeast, will be referred to as 'ethanol extract'.

Fractions of the ethanol extract and the ethanol-extracted yeast cell residues were hydrolysed with 6N-HCl at 105° for 24 hr. HCl was removed *in vacuo* and water added to the residue to give total N concentrations of 5 mg./ml.

The basic technique of paper chromatography was as described by Consden, Gordon & Martin (1944) and Dent (1948), with two-dimensional chromatograms run first in phenol (NH_3 atmosphere) and then in collidine-lutidine as the organic solvents. A modification of Dent's box for running chromatograms was used (Fig. 1); this allows six or eight chromatograms to be run from a single trough by suitable spacing of three or four glass rods on either side of the trough. The point of application (*X* in Fig. 1) of the solution to the paper square must be below the level of the glass rod when the papers are hanging from the trough.

The estimation consisted of finding the amount of amino-acid which gave on a chromatogram a spot of similar size and colour intensity to the corresponding spot obtained from the unknown solution when run under similar conditions (Dent, 1948; Work, 1949). Because of the occurrence of unpredictable variations in chromatograms, even in those run simultaneously, the spots were compared by eye, the accuracy of the method being limited by reproducibility. The error was reduced to ±30% by using the following technique: each pair of spots (standard and unknown) was compared at three intensities, the lowest being the minimum detectable amount and the others twice and four times this amount. The minimum detectable amounts were of the same order as those given by Pratt & Auclair (1948). Owing to wide differences in concentrations of individual amino-acids in the yeasts, amounts of yeast solutions were applied corresponding to levels of total N between 1·3 and 200 μg., ascending in geometrical progression. Repeated comparisons at each level were carried out.

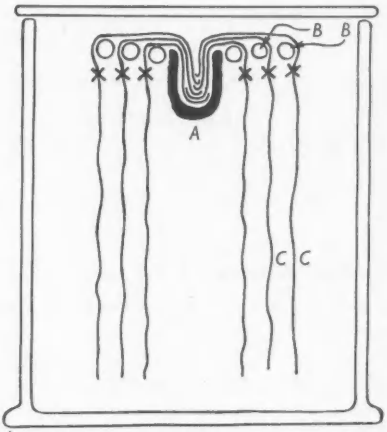

Fig. 1. Sectional diagram of arrangement for running multiple squares. *A*, trough with solvent; *B*, glass rods; *C*, filter-paper square; *X*, point of application of solution for chromatography.

While the majority of amino-acids were examined on two-dimensional chromatograms in the standard way, special techniques were adopted for certain of them. Tyrosine and histidine were estimated after running only in collidine-lutidine, the spots being developed with the Pauly reagent (Dent, 1948). Isoleucine and leucine were separated by using *tert.*-amyl alcohol in the presence of diethylamine vapour (Work, 1949); ornithine, which joins the lysine spot on two-dimensional chromatograms, was separated from lysine by running in the phenol direction in an atmosphere of acetic acid instead of NH_3 and was distinguished from hydroxylysine by its stability to periodate. Methionine and cystine were detected as methionine sulphone and cysteic acid respectively, after oxidation of the solution with H_2O_2 using NH_4^+ molybdate as catalyst (Dent, 1948); methionine and cystine could not be estimated quantitatively because of variable losses, probably caused by peroxide, and were therefore determined gravimetrically. Cystine S was estimated on 2 g. samples by the method of Lugg (1938); methionine S was calculated from the difference between total organic S and cystine S.

Glutathione analysis. The identification of glutathione on paper chromatograms was complicated by the fact that it formed three spots (see Results). These were examined separately by cutting out and eluting (Work, 1949) and the eluates were hydrolysed with 6N-HCl and examined for amino-acids. Although after H_2O_2 oxidation of glutathione

there was only one spot, the method could not be used for estimation, for the same reason as in the case of methionine and cystine. Iodometric titration was therefore used, reduced glutathione being estimated in ethanol-salicylosulphonic acid extracts of whole yeast (Astrup & Øhlenschläger, 1948). Electrolytic reduction of these extracts as described by Dohan & Woodward (1939), followed by iodometric titration, gave the level of total glutathione (oxidized and reduced). The glutathione contents of the ethanol extracts of yeast used for chromatography were also estimated by titration after electrolytic reduction.

RESULTS

Nitrogen distribution in yeast fractions

The nitrogen contents of successive 75% ethanol extracts of baker's yeast are shown in Table 1; four extractions were considered sufficient to remove practically all the soluble nitrogen from the yeast. The figures for the aqueous phase after chloroform treatment of the combined extracts indicate that a threefold concentration was achieved by this process with loss of only 13% of the nitrogen; this nitrogen was probably contained in substances other than amino-acids, since no amino-acids could be detected by chromatography of the acid-hydrolysed contents of the whole chloroform phase from 10 g. of yeast.

Table 1. *Nitrogen contents of successive 75% (v/v) ethanolic extracts from baker's yeast*

(10 g. yeast, 100 ml. 75% (v/v) ethanol in each extraction.)

Extract no.	Volume (ml.)	N content of extract (mg. N/ml.)	N content of extract Percentage of extractable N
1	85	0·682	59·0
2	85	0·284	24·5
3	83	0·113	9·5
4	80	0·086	7·0
Combined	333	0·296	100
Aqueous phase after treatment of combined extracts with 3 vol. of $CHCl_3$	92	0·940	87

Table 2. *Nitrogen distribution in the two yeasts*

	Baker's yeast	Brewer's yeast
Whole yeast (g. N/100 g. yeast)	7·35	8·40
Wt. lost after ethanol extraction (g./100 g. yeast)	28·0	22·0
75% ethanol extract, untreated (g. N/100 g. total N of yeast)	13·5	17·7
75% ethanol extract, 'aqueous phase' after chloroform treatment (g. N/100 g. total N of yeast)	11·7	16·1

Table 2 shows the nitrogen contents of the yeasts and of the various fractions used for chromatography.

Chromatographic estimation of amino-acids

The results of the estimations of amino-acids in the ethanol extracts and in the insoluble residues of the yeasts are shown in Table 3. The figures for whole yeasts were mostly not obtained directly, but were calculated by summing the contents of the hydrolysed ethanol extracts and the insoluble residues.

Diagrammatic representations of typical chromatograms from each fraction are shown in Fig. 2; the level of nitrogen, representing the amount of solution applied to the chromatogram, being chosen to illustrate most clearly the characteristic picture obtained from each extract. Chromatograms of the acid-hydrolysed ethanol-insoluble residues (Figs. 2*A* and 2*a*) showed all the amino-acids normally found in protein hydrolysates, with ornithine present in addition in brewer's yeast. There were no striking differences between the two yeasts, except that baker's yeast had lower contents of arginine and threonine, and a higher content of phenylalanine.

The ethanol extract of baker's yeast (Fig. 2*B*) contained all the amino-acids of the hydrolysed cell residues, with the exception of methionine and cystine, which were not detectable after H_2O_2 oxidation. The relative proportions of the amino-acids bore no resemblance to those of the cell residues. The chromatograms showed in addition the following six spots: nos. 2, 3, 4, 11, 15 and 25. Spots nos. 2, 3 and 4 can be attributed to glutathione, and will be discussed later. Spot no. 11, which was brown in colour, was identified as asparagine; it disappeared after acid hydrolysis. Spot no. 15 may be a peptide with considerable stability to acid hydrolysis; hydrolysed eluates from paper cuts in the position of this spot contained aspartic acid, glutamic acid, glycine and alanine, besides some unchanged material. Spot no. 25 was acid-stable and corresponded in position to γ-aminobutyric acid; its identity was checked by its stability to copper treatment (Crumpler & Dent, 1949). No tryptophan was found in the extract.

The ethanol extract of brewer's yeast, although generally resembling that of baker's, differed from it in certain respects as shown in Table 3 and Fig. 2*b*. The most striking differences occurred in the contents of glutathione, arginine and threonine; α-aminobutyric acid (spot no. 17) was present, although absent from unhydrolysed baker's yeast extracts. The lower

Table 3. *Amino-acid and glutathione contents of yeast fractions*

(The amino-acid contents were estimated chromatographically, except for chemical estimations on S-containing compounds. 0, indicates amino-acid destroyed by hydrolysis; —, indicates amino-acid not detected.)

	Amino-acid content (g. amino-acid N/100 g. N of fraction or of whole yeast)							
	Baker's yeast				Brewer's yeast			
	Ethanol-insoluble residue hydrolysed	Ethanol extract	Ethanol extract hydrolysed	Whole yeast*	Ethanol-insoluble residue hydrolysed	Ethanol extract	Ethanol extract hydrolysed	Whole yeast*
Alanine	10	4	6	9	10	4	6	9
β-Alanine	—	—	0·3	0·04	—	—	—	—
α-Aminobutyric acid	—	—	0·3	0·04	—	0·1	0·3	0·05
γ-Aminobutyric acid	—	0·5	1	0·1	—	0·5	1	0·2
Arginine	5	2·5	5	5	10	8	10	10
Aspartic acid	8	1·5	5	8	8	3	5	7
Asparagine	0	3	0	0·4	0	3	0	0·5
Cystine	0·9†	—	3‡	1·25§	0·9†	—	1·5‡	1·5§
Glutamic acid	10	10	20	11	10	5	20	12
Glutamine	0	—	0	—	0	2	0	0·3
Glycine	8	0·1	3	7	8	0·5	3	7
Histidine	8	2	2	7	8	1	1	7
Isoleucine	4	0·8	0·8	3	4	0·5	0·5	3
Leucine	6	0·8	0·8	5	6	0·5	0·5	5
Lysine	9	2	4	8	9	2	4	8
Methionine	0·7§	—	—	0·83§	0·5§	—	—	0·81§
Ornithine	—	—	—	—	1	5	5	1
Phenylalanine	3	2	2	3	1·6	—	0·7	1
Proline	3	2	2	3	3	2	2	3
Serine	4	0·7	0·7	4	4	0·7	0·7	3
Threonine	5	2	2	4	7	—	1	6
Tryptophan	0	—	0	—	0	—	0	—
Tyrosine	3	1	1	3	2	0·5	0·5	2
Valine	8	2	2	7	8	2	2	7
Sum	96	37	61	90	101	42	65	94
Glutathione	0	9·5	0	1·5‖	0	4·5	0	1·7‖
Total N content of fraction (g./100 g. untreated yeast)	6·36	0·86	0·86	7·35	6·92	1·35	1·35	8·40

* Sum of the values obtained for ethanol-insoluble residue and for hydrolysed ethanol extract.
† Calculated by difference; whole yeast cystine N minus cystine N in whole yeast glutathione.
‡ Cystine N in glutathione of ethanolic extract.
§ Estimated on whole yeast.
‖ Estimated on ethanol-salicylosulphonic acid extract of whole yeast.

glutathione content of the ethanol extract of the brewer's yeast was confirmed by iodometric titration. The unidentified spot no. 15 of the ethanol extract of the baker's yeast was not found, but a weak spot corresponding to glutamine was present.

Acid hydrolysis of the ethanol extracts (Figs. 2*C*, 2*c*) caused marked increases in the strengths of spots given by aspartic acid, glutamic acid, glycine and alanine; cysteic acid appeared. The glutathione, glutamine and asparagine spots disappeared, accounting, in part at least, for the increases noted. In baker's yeast spot no. 15 decreased in strength, and four additional spots appeared: no. 17 (α-aminobutyric acid), no. 18 (β-alanine) and nos. 9 and 20. Spot no. 20 resembled, both in colour (yellow) and in position, the spot given by thiazolidine-4-carboxylic acid (Consden, Gordon & Martin, 1946), but was shown to be different from this substance, as chromatograms run with thiazolidinecarboxylic acid added to the hydrolysate gave two neighbouring yellow spots. Spot no. 20 was not present in hydrolysed brewer's yeast extract. Spot no. 9 which was stronger in chromatograms in hydrolysed brewer's yeast extract than in those from baker's yeast extract, corresponded in position to either diaminopimelic acid (Work, 1950) or ethanolamine-*O*-phosphoric acid. It was shown to be different from diaminopimelic acid by electrodialysis, when it moved with the acidic fraction; it may, therefore, be ethanolamine phosphoric acid, but its identity was not confirmed.

Paper chromatography of glutathione. Pure natural and synthetic glutathione, dissolved in water or 75% ethanol, did not give reproducible chromatograms; usually there were three spots (nos. 2, 3 and 4, Fig. 2*B*), but they were not constant in position or strength. Spot no. 2 was often elongated in the direction of the glycine spot (spot no. 8), and was the only one present after heating an aqueous solution of glutathione for 15 min. at 80°. Spot no. 4 varied from a single spot below aspartic acid (spot no. 5) to a streak between

spots nos. 4 and 3; this streak was sometimes divided into two separate spots. Spot no. 3 was not always present, but after oxidation of the glutathione with H_2O_2 and NH_4^+ molybdate it was the only spot and had much increased in strength. The behaviour of spot no. 3 resembled that of cysteic acid, it was sometimes present after running an ordinary chromatogram, but it was always increased in strength after peroxide oxidation. By analogy with cysteic acid, one may attribute spot no. 3 to an oxidized form of glutathione, possibly containing sulphur as a sulphonic acid group.

Ethanol extracts from baker's yeast gave spots nos. 2–4 which varied in the same way as spots from solutions of pure glutathione. These spots, after elution with water at 2° from several chromatograms of the ethanol extract, we.e examined separately. Running chromatograms of the eluates showed that spot no. 2 gave spots nos. 2 and 3, spot no. 3 gave nos. 3 and 4, while spot no. 4 did not give any one identifiable spot. Hydrolysed eluates from spot no. 3 contained glutamic acid, cysteic acid (from cystine) and glycine, while eluates from spots nos. 2 and 4 contained glutamic acid, glycine and alanine but no cystine. The origin of the alanine in these hydrolysates must be attributed to cystine or cysteic acid. Brewer's yeast extracts produced the same three spots attributable to glutathione, but they were of lower strengths.

Distribution of sulphur and sulphur-containing amino-acids in yeasts

Table 4 summarizes the results on the content and distribution of sulphur in the yeasts. The differences in the total sulphur contents of the two yeasts were greater than those in organic sulphur owing to the virtual absence of sulphate from baker's yeast. The latter contained 0·35% organic sulphur, 60% of which was cystine sulphur. The corresponding values for brewer's yeast were 0·44% organic sulphur and 65% cystine sulphur. Allowing for the cystine content of glutathione, 36% of the organic sulphur of baker's yeast and 40% of that of brewer's yeast may be calculated to be present as protein cystine. Methionine, calculated as the difference between organic sulphur and total cystine sulphur, also makes up roughly similar percentages of organic sulphur in the two yeasts (40 and 35% for baker's

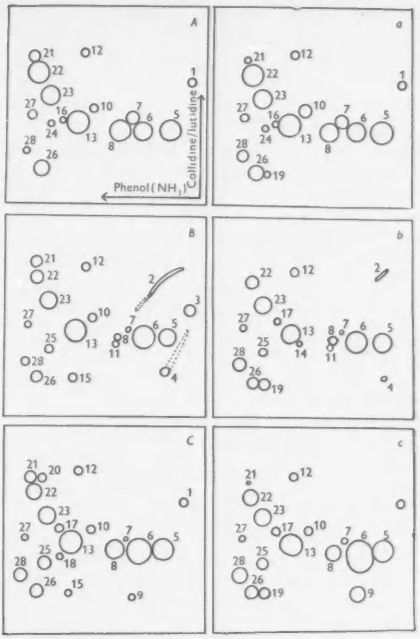

Fig. 2. Chromatograms from yeast fractions. (Solvents: 1, Phenol/NH_3 and 2, collidine/lutidine.) *A*, *B* and *C*, baker's yeast; *a*, *b* and *c*, brewer's yeast. *A*, *a*, acid-hydrolysed ethanol-insoluble residue, equiv. to 50 μg. N. *B*, *b*, ethanol extract, equiv. to 100 μg. N. *C*, *c*, acid-hydrolysed ethanol extract, equiv. to 100 μg. N. 1, Cysteic acid; 2, 3 and 4, glutathione; 5, aspartic acid; 6, glutamic acid; 7, serine; 8, glycine; 9, unknown; 10, threonine; 11, asparagine; 12, tyrosine; 13, alanine; 14, glutamine; 15, unknown; 16, histidine; 17, α-aminobutyric acid; 18, β-alanine; 19, ornithine; 20, unknown; 21, phenylalanine; 22, leucine and isoleucine; 23, valine and methionine; 24, methionine sulphoxide; 25, γ-aminobutyric acid; 26, lysine; 27, proline; 28, arginine.

Table 4. *Sulphur distribution in yeasts*

	Sulphur (g./100 g. dry yeast)		Sulphur (g./100 g. organic S)	
	Baker's yeast	Brewer's yeast	Baker's yeast	Brewer's yeast
Total S	0·36	0·55	—	—
Sulphate S	0·01	0·11	—	—
Organic S	0·35	0·44	—	—
Total cystine S	0·21	0·29	60	65
Total glutathione S (oxidized + reduced)	0·08	0·11	24	25
Protein-cystine S	0·13	0·18	36	40
Methionine S (organic S – total cystine S)	0·14	0·16	40	35
Ethanol-extractable S	0·09	0·08	—	—
Ethanol-extractable glutathione S (oxidized + reduced)	0·06	0·05	17	11
Ratio, total S:total N	1:20	1:15	—	—
Ratio, organic S:total N	1:21	1:19	—	—

and brewer's respectively). Because of the different organic sulphur contents of the two yeasts, the differences in the concentration of the sulphur-containing amino-acids, particularly cystine, become more obvious when the results are expressed in terms of dry yeast.

Extraction of whole yeast with 75% ethanol evidently did not extract all the glutathione, since the ethanol extracts showed lower glutathione contents than did the salicylosulphonic acid extracts. In the salicylosulphonic acid extracts, 71% of the glutathione of baker's yeast and 62% of that of brewer's yeast was present in the reduced form.

When considering the sulphur contents of the yeasts in relation to their total nitrogen contents, the higher nitrogen content of the brewer's yeast is of importance. Because of this, the ratio between organic sulphur and total nitrogen is almost the same in the two yeasts (1:21 and 1:19). The total sulphur:total nitrogen ratios differ (1:23 and 1:15), due to the high sulphate sulphur content of brewer's yeast.

DISCUSSION

Our figures for the amino-acid composition of the yeasts are based mainly on a paper-chromatographic method of estimation. This method, although not accurate (±30%), is very specific and has the advantage of being applicable to nearly all amino-acids except cystine and methionine. Acid hydrolysis was used to split the protein, and so tryptophan was not estimated, since it is destroyed by this treatment.

Various methods have been suggested for the quantitative estimation of amino-acids by paper chromatography (Block, 1948; Fisher, Parsons & Morrison, 1948; Woiwod, 1948; Naftalin, 1948; Martin & Mittelmann, 1948; Polson, 1948; Landua & Awapara, 1949). With the exception of those of Block and Polson, these methods are only applicable to simple amino-acid mixtures and could not be used in the present case (see Martin, 1949). The methods of Block and Polson are essentially similar to those employed here, except that Block makes his measurements with mechanical aids. Polson claims greater accuracy (±10%) than we have achieved and has given his figures for the amino-acid content of *Escherichia coli* to two places of decimals. Neither of these authors emphasized the necessity of using very pale spots near the minimal detectable level for comparison, a condition which we found essential.

Cystine and methionine, of particular interest in liver necrosis, were estimated gravimetrically (Lugg, 1938) because both chromatographic and microbiological methods of estimation proved unreliable. In the gravimetric method, the assumption is made that cystine and methionine comprise the bulk of the organic sulphur components. This assumption, although justifiable for pure proteins, cannot be strictly correct in the case of yeast, which is known to contain small amounts of other sulphur-containing compounds. The extent to which the presence of these compounds influenced our results is unknown; we are only able to say that taurine, which is detectable on chromatograms, was not found in our yeasts. A justification for our use of the method lies in the fact that the figures obtained for methionine are not unduly high, and so could not have been much affected by other sulphur-containing compounds not oxidized under conditions used in Lugg's method.

Published figures for the amino-acid contents of yeasts show some variations between different strains, but no gross or regular differences between baker's and brewer's yeast. Table 5 gives a comparison of our results with some of those reported in the recent literature obtained by chemical or microbiological methods. The present method of estimation is the only one which has produced figures for all the amino-acids present except tryptophan. Attention should be drawn to the differences between the results reported here on the sulphur, cystine and methionine contents of baker's yeast and those of Dent (1947), estimated by the same method on a different batch sample of the same strain of yeast. The main difference lies in the level of organic sulphur, which was much higher (0·5 g. sulphur/100 g. yeast) in Dent's sample; since methionine was estimated by difference, the methionine content was consequently also higher. It is now obvious that analytical figures are not identical on different batches of the same strain of yeast; György's figures for sulphur and nitrogen in both our baker's and his brewer's yeasts also differ from our figures (Table 5). A possible cause of these differences could be variations in the composition of the growth medium for the yeast: D.C.L. baker's yeast is grown on molasses, a substance whose composition might be expected to be relatively inconstant. Barton-Wright (1949) reports that the methionine content of baker's and brewer's yeasts can be altered by changes of the methionine content of the growth medium. It is therefore possible that variations in the organic sulphur content of the D.C.L. baker's yeast could, in part, be attributed to variations in methionine content.

Chromatograms from the ethanol-insoluble cell residues of both yeasts gave no unidentifiable spots, nor even any unusual ones except for small amounts of ornithine in brewer's yeast. The ethanol extracts contained all the amino-acids of the cell residues, except for cystine and methionine. They also contained α- and γ-aminobutyric acids, amino-acids which have so far not been identified in proteins, but are known to occur in extracts from animals and plants (Dent, 1948) and from bacteria (Work, 1949 and unpublished observations). Baker's yeast

Table 5. *Comparison of analytical results on yeasts with published values*

(Except where stated, figures are calculated as g. of amino-acid N/100 g. total N)

Type of yeast ...	Baker's D.C.L.			Baker's	Brewer's (Fleischmann's)		Brewer's		
Author and reference	Present results	Dent (1947)	György (1948)	Block & Bolling (1945)	Present results	György (1948)	Block & Bolling (1945)	Edwards *et al.* (1946)	Barton-Wright (1949)
Method of estimation	Chromatographic, *Chemical	Chemical	—	Chemical, †Microbiological (mean of two values)	Chromatographic, *Chemical	—	Chemical, †Microbiological (mean of three values)	Chemical (mean of two values)	Microbiological
Arginine	5	—	—	7·5	10	—	10	26·2	—
Cystine	1·3*	1·0	—	0·7	1·5*	—	0·7	—	—
Histidine	7	—	—	4·8	7	—	4·8	5·0	4·5
Isoleucine	3	—	—	4·1†	3	—	3·9†	4·0	3·0
Leucine	5	—	—	4·8†	5	—	5·1†	5·2	3·7
Lysine	8	—	—	8·7	8	—	8·4	8·75	8·2
Methionine	0·8*	2·0	—	1·6	0·8*	—	1·6	1·3	0·9
Phenylalanine	3	—	—	2·2	1	—	2·3	1·8	1·8
Threonine	4	—	—	4·2	6	—	4·1	3·7	—
Tryptophan	—	—	—	1·4	—	—	1·6	0·6	—
Tyrosine	3	—	—	1·8	2	—	1·7	3·1	—
Valine	7	—	—	3·5†	7	—	3·5†	—	4·8
Total N, g./100 g. yeast	7·4	7·0	6·8	7·2	8·4	7·8	8·1	—	—
Total S, g./100 g. yeast	0·36	0·53	0·65	0·36	0·55	0·86	0·4	—	—
Ratio, total S:total N	1:20·6	1:13·2	1:10·5	1:20	1:15·3	1:9·1	1:20·3	—	—

ethanol extract showed an unknown spot (no. 15) which may be an acid-stable peptide. After hydrolysis, the ethanol extract gave in addition spots from β-alanine, ethanolamine-*O*-phosphoric acid (spot no. 9, not confirmed rigidly) and an unidentified substance (spot no. 20). Ethanolamine phosphoric acid was present in higher concentrations in hydrolysed ethanol extract from brewer's yeast.

Both ethanol extracts contained glutathione, which gave multiple spots, not yet fully identified. Each spot after elution hydrolysed to glutamic acid, glycine and cystine or alanine (derived from cystine), but the proportions of the amino-acids in each fraction were not determined. The streaky nature of two of the spots suggests that they were caused by changes occurring during development of the chromatogram. The reason is unknown for the higher glutathione contents of the salicylosulphonic acid extracts of the yeasts compared with the ethanol extracts. The iodometric titration method may estimate other reducing substances in addition to glutathione. Unfortunately, no independent check on glutathione content by glyoxalase coenzyme estimation could be made, because after neutralization of the salicylosulphonic acid extract the titration figure dropped rapidly—owing probably to oxidation of the glutathione. The considerable difference (30% or more) in the levels of total and reduced glutathione do not agree with the findings of Astrup & Øhlenschläger (1948), who state that the titration figure never rose by more than 10% after electrolytic reduction.

The conclusions we can draw about the composition of baker's and brewer's yeasts are that the brewer's yeast is about 14% richer in protein, if we assume that a similar proportion of the total nitrogen of each sample is non-protein nitrogen. Weight for weight, brewer's yeast is also richer in the sulphur-containing amino-acids, having 38% more total cystine and 14% more methionine than baker's yeast. The same proportion of total cystine is represented by glutathione in both yeasts, therefore brewer's yeast contains 38% more glutathione than baker's yeast. If their amino-acid compositions calculated on the basis of total nitrogen are compared, the yeasts appear to be roughly similar except that brewer's yeast contains more arginine and threonine and less phenylalanine than baker's yeast.

SUMMARY

1. One sample of baker's yeast and one sample of brewer's yeast were compared with regard to their contents of nitrogen, sulphur, amino-acids and glutathione.

2. Separate estimations were made on the 75% ethanol extracts and on the ethanol-insoluble residues.

3. Some modifications of the technique of estimating amino-acids by paper chromatography were used.

4. The behaviour of glutathione on paper chromatograms is described.

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The Nutritive Properties of Two Yeasts used to Produce Massive Dietetic Liver Necrosis in Rats

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Dietetic liver necrosis in rats has been produced by feeding diets deficient both in protein and in tocopherol (Himsworth, 1950). Such diets containing suboptimal amounts of casein, soya-bean meal or yeast, produce liver necrosis within periods varying from a few weeks to several months; when extra cystine or methionine is added to the diets the lesion is prevented (Weichselbaum, 1935; Hock & Fink, 1943*a*; Himsworth & Glynn, 1944*b*; Matet, Matet & Fridenson, 1947; György & Goldblatt, 1949). A deficiency in the sulphur-containing amino-acids relative to other amino-acids in the diet thus appears to be one of the factors responsible for the development of liver necrosis. This theory is supported by experiments of Glynn, Himsworth & Neuberger (1945) in which dietetic liver necrosis was produced in rats by substituting the dietary protein by a synthetic mixture containing all the essential amino-acids except methionine and cystine. Hock & Fink (1943*b*), following an investigation of the necrogenic properties of diets in which the major part of the protein was provided by different types of yeasts, associated the ease of development of liver necrosis with the relative amounts of sulphur and nitrogen in the diet. When the sulphur:nitrogen ratio was 1:30 the incidence of necrosis was high, but there was no necrosis with a ratio of 1:13. It is obvious that the authors thought that this ratio

might be a rough measure of the amounts of the sulphur-containing amino-acids present in the dietary protein.

In this laboratory, diets containing 18% yeast as the sole source of protein (approx. 7% protein) have been used to produce liver necrosis (Himsworth & Glynn, 1944*a*), but comparison of the results with those obtained by György & Goldblatt (private communication and 1949) on their yeast diets showed that their incidence and severity of necrosis was much lower. The yeast used in this laboratory was baker's yeast, while György used a brewer's yeast. Since it was probable that the difference in types of yeast accounted for the dissimilar results in the two laboratories, samples of yeasts were exchanged in order to compare their nutritional properties for the same strain of rat and also their chemical composition. György's results showed no great difference between the two yeasts with respect to their total S:N ratios (1:10·5 for our baker's and 1:9·1 for his brewer's yeast), but there was a marked difference in their necrogenic properties; thirteen out of fourteen rats on our baker's yeast diet died of acute liver necrosis, while none of fourteen rats fed György's brewer's yeast developed any lesion (György, 1948).

Determination of the amino-acid compositions of the two yeasts is reported in the preceding paper (Lindan & Work, 1951); this paper describes a comparison of their dietetic properties. The necrogenic properties of yeast diets were compared using young tocopherol-depleted rats and the biological values of the yeast proteins were studied by growth experiments on young normal rats. Following the observation of Leaf & Neuberger (1947) that a deficiency of sulphur-containing amino-acids lowered the level of liver glutathione, the effect of the two yeast diets on liver glutathione was also investigated. A connexion was sought between the nutritional differences shown by the two yeasts and their content of amino-acids, particularly the sulphur-containing amino-acids. No investigations on the tocopherol contents of the yeasts were carried out, since tocopherol is supposed to be absent from all types of yeast (György, 1948, and private communication).

EXPERIMENTAL

General. The yeast used in this laboratory to produce rat liver necrosis was a dried baker's yeast (D.C.L. no. G.B. 161) marketed by Distiller's Co. Ltd. London. The sample provided by Dr P. György, was a dried brewer's yeast (Fleischmann's type 50*B*) marketed by Standard Brands, Inc., U.S.A. All our nutritional experiments were carried out with the same bulk samples of each yeast; these were also used for the analyses reported in the previous paper (Lindan & Work, 1951).

The composition of the diets (Himsworth & Glynn, 1944*b*) was as follows: dried yeast (baker's or brewer's), 18%; corn starch, 73%; salt mixture (Glaxo), 3%; olive oil (B.P.), 5%; cod liver oil, 1%. (The dried yeast contained about 6% moisture.)

The olive oil and cod liver oil were mixed daily into the diets, as were the following vitamins, the figures given being the amount allowed for each rat: aneurin, 20 μg.; riboflavin, 25 μg.; pyridoxine, 20 μg.; calcium pantothenate, 100 μg.

Newly weaned albino rats (wt. 34–46 g.) were used as experimental animals. They were of the same strain (Wistar) as used in other experiments on dietetic liver necrosis in this laboratory (Lindan & Himsworth, 1951) and were kept three to five together in cages with raised wire-mesh bases.

Experiment on necrogenic properties of yeasts. Tocopherol-depleted animals were used for this experiment in order to eliminate the protective action of stored tocopherol against liver necrosis (Himsworth & Lindan, 1949). The young rats and their mothers were fed a diet low in tocopherol (approx. 1·1 mg. tocopherol/100 g. diet) from the tenth day of lactation until weaning at 21 days (Mason & Bryan, 1938; Lindan & Himsworth, 1951). Eighteen such weanling rats from three litters were divided into two groups, the first group (five rats) receiving baker's, the second group (thirteen rats) brewer's yeast diet. The diets were fed *ad lib.* for 64 days; after that the seven surviving rats, which all belonged to the second group, were subdivided into two groups and given either baker's or brewer's yeast diet restricted to 8 g./rat/day. This restriction of food intake was found by Glynn & Himsworth (1944) to be necessary to produce necrosis in rats large enough to consume more than this amount of food when food consumption was unrestricted.

Experiment on growth-promoting properties of yeasts. The weanling rats used were reared on normal stock diet. In order to avoid early deaths from necrosis they were not tocopherol-depleted. Nine litter mates were divided into three groups, three rats to a cage, and the food consumption of each group was recorded daily. Baker's and brewer's yeast diets were fed *ad lib.* to groups 1 and 2 respectively. Because more brewer's yeast diet was consumed than baker's, the third group was fed brewer's yeast diet in amount restricted to that consumed by group 1 receiving baker's yeast. The experiment was continued for 33 days, after which the rats were killed and their livers were analysed in order to determine the influence of diet on liver composition. The values for reduced glutathione (iodometric method of Leaf & Neuberger, 1947), total N and water contents were compared with those of normal rats.

RESULTS

Necrogenic properties of yeasts

Growth curves of eighteen tocopherol-depleted rats fed the diets containing either baker's or brewer's yeast as the sole source of protein are shown in Fig. 1. The occurrence of each death from acute liver necrosis is also indicated.

The five rats fed baker's yeast diet *ad lib.* showed no gain in weight and they all died of acute massive liver necrosis after 24–30 days (mean 26 days) on the diet. The general condition of the animals and the histological picture of their livers corresponded with those found in other experiments on liver necrosis from this laboratory (Glynn & Himsworth, 1944; Lindan & Himsworth, 1951).

The brewer's yeast diet fed *ad lib.* for 64 days supported a fair rate of growth (1·2 g./day) and only six out of thirteen rats died of necrosis between 28 and 45 days (mean 35 days). The rats which died had not shown any difference in growth rate compared with those which survived for this period. The pathological changes in their livers (acute massive necrosis) were similar to those found in the necrotic

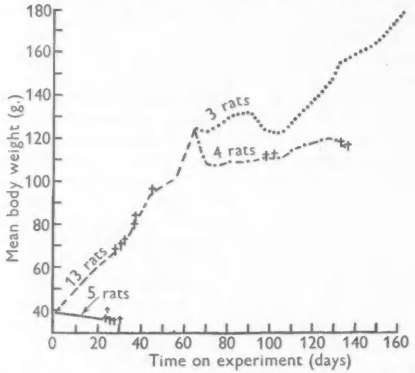

Fig. 1. Average growth curves and occurrence of liver necrosis in tocopherol-depleted rats fed yeast diets. ——, Baker's yeast diet *ad lib.*; – – –, brewer's yeast diet *ad lib.*; ·—·—·, baker's yeast diet 8 g./rat/day; ·····, brewer's yeast diet 8 g./rat/day; +, death from acute liver necrosis.

rats fed baker's yeast diet, but the animals' general condition was far better. The remaining seven animals continued to grow at the same rate until the 64th day. After that, three rats were continued on brewer's yeast diet, but in amounts reduced to 8 g./rat/day. These animals survived another 96 days in fairly good condition; when killed they showed neither liver necrosis nor post-necrotic scarring. The diet of the other four rats was changed to one containing baker's yeast and their food intake was restricted to 8 g./day; all died of liver necrosis within 34–74 days. Even after restriction of the food intake, the superior nutritive properties of the brewer's yeast were revealed by the growth curves.

Growth-promoting properties of yeasts

The results of feeding yeast diets to rats not depleted of tocopherol are shown in Fig. 2, where mean growth curves and food consumption are recorded. There were no deaths from necrosis during the experimental period of 33 days. During the whole experiment, the rats fed brewer's yeast diet *ad lib.* consumed 48 % more food than did the ones receiving baker's yeast. After about 12 days, the food consumption of both groups decreased, by 12 % in the case of brewer's yeast (group 2), and 31 % in group 1 on baker's yeast. The growth curves

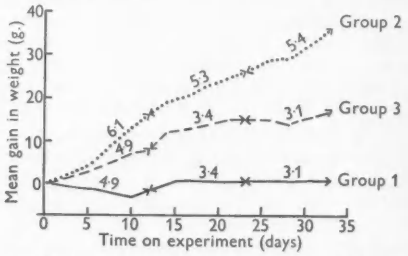

Fig. 2. Effect of yeast diets on growth of weanling rats (mean initial wt. 40 g.). Each curve represents mean values for three rats. ——, Group 1, baker's yeast diet *ad lib.*; ····, group 2, brewer's yeast diet *ad lib.*; – – –, group 3, brewer's yeast diet restricted to consumption of group 1. Figures against curves indicate average daily food consumption (g./rat) for the periods indicated by arrows.

of groups 1 and 2 fed the two diets *ad lib.* were similar to those of corresponding groups of tocopherol-depleted rats shown in Fig. 1. Group 3, receiving the brewer's yeast diet restricted in amount to that consumed by group 1 on baker's yeast, showed a growth rate of 0·5 g./day which was higher than that of group 1, confirming the higher biological value (weight for weight) of brewer's yeast.

The influence of the diets on the glutathione, total nitrogen and water contents of the livers is shown in Table 1. The livers of all the rats fed the yeast diets showed only half the glutathione content of normal control livers, but there was no difference between

Table 1. *Effect of yeast diets on glutathione, nitrogen and water contents of liver*

(Figures represent mean values for three rats.)

Diets and rats used	Body wt. (g.)	Reduced glutathione (mg./100 g. liver)	Reduced glutathione (mg./100 g. liver N)	Nitrogen (g./100 g. liver)	Water (g./100 g. liver)
Stock diet:					
Weanlings	38	197	68	2·9	72
33 days after weaning	109	207	66	3·1	71
Yeast diets:					
Baker's *ad lib.*	45	93	38	2·4	66
Brewer's *ad lib.*	75	96	35	2·8	66
Brewer's restricted	59	97	41	2·4	67

the three groups of rats on the yeast diets. Both the nitrogen and water contents of the livers of rats fed the yeast diets were slightly lower than those of the controls.

DISCUSSION

It is evident that baker's and brewer's yeasts when used as sources of dietary protein for rats, differ in their nutritive qualities as shown both by their tendency to produce liver necrosis and by their growth-promoting properties. The experiment in which liver necrosis was produced between the 24th and 30th day in the whole of the small group of tocopherol-depleted weanling rats fed diets containing 18% baker's yeast has been confirmed by further data (Lindan & Himsworth, 1951) on 124 rats, all of which developed necrosis within a mean period of 28 days (S.D. ± 10·1). Only half the animals fed brewer's yeast diet developed necrosis, and the time of onset was delayed to between the 28th and 45th day. The relative difference in the necrogenic properties of the two yeasts is similar to the results reported by György (1948), although György found the onset of necrosis with our baker's yeast to be very much later (100th to 144th day, one rat surviving 200 days); his sample of brewer's yeast produced no necrosis within this period. The differences between György's results and ours could be attributed to his using older rats with no pre-experimental depletion of tocopherol. Older rats have been shown to be more resistant to necrosis, and the onset of necrosis may be considerably delayed by feeding diets high in tocopherol in the pre-experimental period (Lindan & Himsworth, 1951). On the other hand, the difference in results might be due to variations in composition of the particular batches of yeast used in the two laboratories; thus the values reported by György for both nitrogen and sulphur differ from our results, particularly in the case of sulphur, which in the baker's yeast is almost double our figure (see Table 5, Lindan & Work, 1951). However, the general conclusion which can be drawn from these two sets of nutritional experiments is that both the baker's yeast samples were much more necrogenic than the brewer's yeast samples.

Comparison of the biological values of the two yeast proteins, judged by growth-promoting properties, agreed with the results of our necrosis experiment. With the same food intake, brewer's yeast as protein produced a slight gain in weight (17 g. in 33 days), while the weights of rats fed the baker's yeast diet remained stationary. With unrestricted feeding appetite was much better in the brewer's yeast group, but food consumption dropped during the experiment, the decrease in the case of baker's yeast diet being more marked. The assumption of Hock & Fink (1943*b*) that the total sulphur: nitrogen ratio in low-protein diets bears a relation to the incidence of liver necrosis should be considered in the light of the following comments. As noted (Lindan & Work, 1951, Table 5), analytical figures for different samples of the same strain of yeast are very variable, ranging from 1:10 to 1:21 for our baker's yeast and from 1:9 to 1:15 for György's brewer's yeast, but the baker's yeast was always more necrogenic than the brewer's. The total sulphur: total nitrogen ratio can give little indication of the relative biological values of the yeasts because the figures for neither sulphur nor nitrogen can be directly related to protein content. Variable proportions of total sulphur consist of sulphate sulphur. This sulphate sulphur probably bears a direct relationship to the sulphur content of the original growth media of the yeasts (Fink & Just, 1939), which may vary from batch to batch.

Considering the relationship between organic sulphur and total nitrogen, the ratios for the two yeasts were found to be very similar (1:21 for baker's and 1:19 for brewer's) and so bore no relation to the necrogenic properties of the yeasts. It is interesting to note that these organic sulphur:nitrogen ratios are of the same order as those (1:18 to 1:20) reported by Fink & Hock (1947) for their yeast diets of variable necrogenic properties.

The total nitrogen figures on which the sulphur: nitrogen ratios are based do not reflect the nutritive values of the yeast proteins, since the amount of non-protein, non-amino-acid nitrogen is not considered. The usual estimation of non-protein nitrogen by trichloroacetic acid precipitation has no significance nutritionally, since a considerable proportion of the non-protein nitrogen must be due to amino-acids or peptides. We found that the proportion of total nitrogen of the yeasts soluble in 75% ethanol was 13% for baker's and 18% for brewer's yeast; of this amount about 63% was represented by amino-acid nitrogen, as determined chromatographically (Tables 2 and 3, Lindan & Work, 1951). It appears then that about 5 and 7% of the total nitrogen was extractable non-amino-acid nitrogen in the baker's and brewer's yeast respectively. The amount of ethanol-insoluble non-protein nitrogen, also calculated by chromatography, was about 10 and 6% of the total nitrogen respectively. The total non-protein non-amino-acid nitrogen can therefore be assumed to be about 14% of the total nitrogen of both yeasts. As brewer's yeast contained 8·40% total nitrogen, compared with 7·35% for baker's yeast, we can conclude that, weight for weight, the former yeast had more protein.

Our diets containing 18% of baker's and brewer's yeast were intentionally very poor in protein (about 7 and 8% respectively, calculated as protein nitrogen × 6·25). Table 2 shows the minimal requirements of the essential amino-acids for normal growth in

Table 2. *Essential amino-acid composition of necrogenic yeast diets compared with minimal requirement for normal growth of rats, after Rose* (1937)

(Columns 4–6 represent total average amino-acid intake per rat for 33 days found in the growth experiment for the three groups.)

	Amino-acid, mg. in					
	100 g. diet			123 g. diet		180 g. diet
	Rose's minimal amount for normal growth	Baker's yeast	Brewer's yeast	Baker's yeast (group 1)	Brewer's yeast (group 3)	Brewer's yeast (group 2)
	(1)	(2)	(3)	(4)	(5)	(6)
Arginine	200	190	440	230	540	790
Histidine	400	320	370	390	450	660
Isoleucine	500	350	400	430	490	720
Leucine	800	580	670	710	820	1210
Lysine	1000	520	590	640	730	1060
Methionine	600	110	120	135	150	220
Cystine	—	130	180	160	220	320
Phenylalanine	700	440	170	540	210	310
Threonine	500	430	720	530	880	1300
Tryptophane	200	—	—	—	—	—
Valine	700	730	830	900	1020	1490

rats, as given by Rose (1937), compared with the essential amino-acid composition of the yeast diets used in the present work and based on figures reported (Lindan & Work, 1951; Table 3). Besides a marked deficiency in sulphur-containing amino-acids in both diets, particularly in baker's yeast diet, the table shows that other amino-acids, notably phenylalanine, lysine and leucine were below the minimum figures suggested by Rose. Although addition of extra cystine or methionine to yeast diets is known to afford complete protection against liver necrosis, we do not know whether the imbalance among the other amino-acids is also playing a role in the production of necrosis. The total average consumption per rat of each essential amino-acid during the whole period of the growth experiment (Fig. 2) is also given in Table 2. All the essential amino-acids except phenylalanine were consumed in larger amounts by the rats given brewer's yeast diet either *ad lib.* or in amounts restricted to the consumption of the baker's yeast diet. This could easily explain the higher growth rate observed with the brewer's yeast diet. Supplementation of baker's yeast diet with cystine up to 0·63 g./100 g. of diet did not improve growth of young rats, although it protected the animals against liver necrosis (Lindan & Himsworth, 1951). The failure of baker's yeast diet to support growth could therefore be due to multiple amino-acid deficiencies.

The two yeasts showed a difference in their contents of ethanol-extractable amino-acids, 0·44 g. of amino-acid nitrogen being extractable from 100 g. of baker's yeast and 0·92 g. from 100 g. of brewer's yeast. The amino-acid make-up of the extracts differed in the case of only a few amino-acids. The nutritional significance of these extractable amino-acids was not assessed in our experiments, but the higher total glutathione content of brewer's yeast might be of importance.

The brewer's yeast was a non-fermenting sample, while the baker's yeast was capable of fermentation. Experiments (Himsworth & Lindan, unpublished observations) with autoclaved (20 min. at 115°) non-fermenting baker's yeast showed that it had not only retained, but even increased its necrogenic properties; twelve animals died within a mean time of 16 days from the beginning of the experiment (S.D. ± 2·9). Thus the difference in the necrogenic properties of the two yeasts was not related to their viability.

Since the liver seems to be the organ which is most affected by the deficiency of cystine and methionine, it was of interest to investigate the effect of yeast diets on the distribution of sulphur in the liver. Lee & Lewis (1934) found that the liver protein composition was normal in rats fed cystine-deficient diets, while Dent (1947) reported no change in the cystine and methionine contents of the liver proteins even when the rats had developed necrosis on baker's yeast diet. The only fraction of liver sulphur which varies appears to be the non-protein fraction and may be attributed to variation in glutathione (Leaf & Neuberger, 1947). Despite differences in the necrogenic and growth-promoting properties of the baker's and brewer's yeast, and in their cystine content, we found that they both produced a lowering of the liver glutathione content from 200 to 95 mg./100 g. liver when fed for 33 days to rats which were not tocopherol-depleted during the lactation period. We have found (unpublished work)

that necrotic livers from tocopherol-depleted rats fed baker's yeast diet had an even lower glutathione content of about 25 mg./100 g. liver.

In conclusion, the nutritive properties of baker's and brewer's yeasts differ in respect to both growth-promoting and necrogenic properties. The amino-acid contents of the two yeasts could explain the difference in growth-promoting properties, the diet containing baker's yeast being the more deficient in several amino-acids. We cannot, however, say whether the small differences in the sulphur-containing amino-acid content account entirely for their different necrogenic properties. Further experiments are needed to establish this point.

SUMMARY

1. A comparison was made between the dietetic properties of British baker's yeast and American brewer's yeast, both of which were used as the sole source of protein in the production of dietetic liver necrosis in rats. Diets containing 7–8 % of yeast protein were used.

2. When fed to tocopherol-depleted weanling rats the baker's yeast diet proved to be more necrogenic than the brewer's yeast diet. Growth experiments on normal weanlings also showed the inferior biological value of baker's yeast.

3. Rats fed the yeast diets for 33 days had liver glutathione contents which were 50 % lower than those of normal rats. There was no difference between the composition of livers of rats fed either type of yeast.

4. Different dietetic deficiencies exhibited by the two yeasts are discussed in relation to their contents of sulphur, nitrogen and amino-acids. Brewer's yeast contains 38 % more cystine and glutathione than baker's yeast. Comparison with Rose's (1937) figures for minimum essential amino-acid requirements for normal growth showed that both yeast diets were also deficient in other essential amino-acids.

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The Use of 'CO_2 Buffers' in Manometric Measurements of Cell Metabolism

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Pardee (1949) has recently devised a simple manometric method for the measurement of the oxygen absorption of cells respiring in the presence of carbon dioxide. The method is generally applicable to systems where carbon dioxide and a second gas are formed or used and where the pressure changes of the second gas are to be measured. It is based on the use, in a centre well of the manometric vessel, of a 'CO_2 buffer' capable of maintaining a virtually constant pressure of carbon dioxide in the gas phase. A suitable 'CO_2 buffer' is an aqueous solution of diethanolamine which binds carbon dioxide reversibly, mainly according to the equation

$$NH(CH_2.CH_2OH)_2 + CO_2 + H_2O \rightleftharpoons HCO_3^- + NH_2(CH_2.CH_2OH)_2.$$

Any carbon dioxide formed by metabolic processes is removed by this reaction proceeding from left to right, and any carbon dioxide used is replaced by the reverse process. Warburg (1919) was the first to use this principle in the form of carbonate-bicarbonate buffer; but only very low carbon dioxide pressures of the order of a few mm. mercury can be maintained by this system, whilst diethanolamine can maintain carbon dioxide pressures of 25 and more mm. mercury.

In applying Pardee's method a complication was encountered which led to a fuller study of the principle. It was observed that solutions of diethanolamine, even after repeated recrystallizations of the amine, absorb oxygen. This raised the question whether the autoxidation was a factor in Pardee's results. The effectiveness of diethanolamine and similar reagents as carbon dioxide buffers was therefore systematically examined. In addition the theoretical basis of the method was studied.

Theory of 'CO_2 *buffers*'

Calculation of buffering capacity. To calculate the capacity of a CO_2 buffer it is necessary to know the relationship between the CO_2 pressure and the concentration of bound CO_2 in the solution. This relationship can be calculated in simple systems based on the reaction

$$\text{monoacidic weak base} + CO_2 \rightleftharpoons HCO_3^- + \text{monoacidic base cation}, \quad (1)$$

if the electrolytic dissociation constant of the base (K_B), the apparent first electrolytic dissociation constant of carbonic acid ($K_{H_2CO_3}$), the dissociation constant of water (K_W), the total concentration of the base (undissociated base + cation) c, the Bunsen absorption coefficient of CO_2 for the solution (α_{CO_2}) are known and if some simplifying assumptions are made, namely that no other reactions between the base and CO_2 (especially no formation of carbamate) occur and that the second stage of dissociation of carbonic acid is negligible.

It follows from the equations defining K_B, $K_{H_2CO_3}$, K_W and from the assumption that, in accordance with reaction 1, the concentration of bicarbonate in the solution equals that of the base cation, that

$$[HCO_3^-] = \sqrt{\left\{\frac{cK_BK_{H_2CO_3}[CO_2]}{K_W} + \left(\frac{K_BK_{H_2CO_3}[CO_2]}{2K_W}\right)^2\right\}} - \frac{K_BK_{H_2CO_3}[CO_2]}{2K_W}, \quad (2)$$

where the square brackets refer to the concentrations in the solution. With this formula the bicarbonate concentration of a base solution can be calculated for any CO_2 pressure. If a salt of a weak acid which reacts with CO_2 according to the equation

$$\text{Anion}^- + CO_2 + H_2O \rightleftharpoons HCO_3^- + \text{acid}$$

is used instead of the base solution, $[HCO_3^-]$ can be calculated by substituting K_{acid} for K_B/K_W in equation 2.

The validity of equation 2 has been tested experimentally for dilute solutions of triethanolamine, and the agreement between calculated and measured data was found to be satisfactory (see p. 353). However, in the concentrations of 3 or 4 M strength, required for buffering purposes, major deviations from the theory occur which are due to the occurrence of significant quantities of carbonate and of carbamate and to additional factors not operative in dilute solution. Mason & Dodge (1936), who have measured the bound CO_2 of concentrated amine solutions at different CO_2 pressures, have come to the conclusion that the observations cannot be accounted for in terms of a simple theory.

Warburg (1925) has shown how data correlating the increase in CO_2 pressure with the increase in bound CO_2 in the solution can be used for calculating manometric pressure changes due to CO_2 in the presence of substances combining with CO_2. In accordance with Warburg's terminology the binding of CO_2 by the solution is referred to as 'retention' of CO_2. If d [retained CO_2] is the increase in the concentration of retained CO_2 (in μl./ml.) produced by the increase in CO_2 pressure dp_{CO_2} (in mm. of manometric fluid), then $\frac{\text{d [retained } CO_2]}{dp_{CO_2}}$ is the 'retention' of CO_2/mm. change in CO_2 pressure/ml. of solution.

Assuming, by first approximation, that

$$\frac{\text{d [retained } CO_2]}{dp_{CO_2}}$$

is, within a limited range of p_{CO_2}, practically constant, the pressure changes in a manometric vessel due to the formation or disappearance of CO_2 can be calculated by the formula

$$h_{CO_2} = \frac{x_{CO_2}}{k_{CO_2} + v_F \frac{\text{d [retained } CO_2]}{dp_{CO_2}}},$$

where h_{CO_2} is the pressure change (in mm.) produced by the amount x_{CO_2} (in μl.), k_{CO_2} the vessel constant for CO_2 and v_F the volume of the retaining fluid (in ml.). It will be seen that for a given value of x_{CO_2} the value for h_{CO_2} will be the smaller, i.e. the retention will become the more effective, the larger v_F; further that for a given value of $\frac{\text{d [retained } CO_2]}{dp_{CO_2}}$ the percentage retention

$$\frac{v_F \frac{\text{d [retained } CO_2]}{dp_{CO_2}}}{v_F \frac{\text{d [retained } CO_2]}{dp_{CO_2}} + k_{CO_2}} \times 100,$$

will be the greater the smaller k_{CO_2}. The ideal system for retaining CO_2 is one in which the above term approaches the value of 100.

In dilute solutions of an amine d [retained CO_2] is practically identical with d [HCO_3^-], and $\frac{d\,[HCO_3^-]}{d[CO_2]}$ can be calculated by differentiating equation 2 with respect to [CO_2]:

$$\frac{d\,[HCO_3^-]}{d\,[CO_2]} = \frac{\frac{cK_BK_{H_2CO_3}}{K_W} + \left(\frac{K_BK_{H_2CO_3}}{K_W}\right)^2 \frac{[CO_2]}{2}}{2\sqrt{\left\{\frac{cK_BK_{H_2CO_3}[CO_2]}{K_W} + \left(\frac{K_BK_{H_2CO_3}[CO_2]}{2K_W}\right)^2\right\}}} - \frac{K_BK_{H_2CO_3}}{2K_W}. \quad (3)$$

[HCO_3^-] and [CO_2] are here expressed in the same units as the dissociation constants which are customarily molar. For manometric work it is convenient to use different units, namely μl./ml. for [HCO_3^-] and mm. of manometric fluid for [CO_2]. It follows from the definition of the Bunsen absorption coefficient α, and the value for the molar volume of CO_2 (22·26 l.), that a CO_2 concentration of 1 M is equivalent to a CO_2 pressure of $222{,}600/\alpha$ mm. manometric fluid. The conversion factor for transforming

$$\frac{\text{g. mol. } HCO_3^-/\text{l.}}{\text{g. mol. } CO_2/\text{l.}} \text{ into } \frac{\mu\text{l. } HCO_3^-/\text{ml.}}{\text{mm. } CO_2}$$

is thus $\frac{22{,}260}{222{,}600/\alpha} = 0{\cdot}1\,\alpha$. The conversion factor obviously depends on the temperature, solute concentration and other factors which affect the value of α.

Equation 3 allows the calculation of the retention at varying CO_2 concentration for the conditions to which the simplifying premises used in the derivation of equation 2 apply. Although the conditions in the solution actually used are more complex, it is of interest to calculate how changes in the values of the constants and of the CO_2 pressure affect the retention. ←

Effect of [CO_2] *on retention.* When [CO_2] is very small all terms in equation 3 containing [CO_2] become very small and the numerator, which contains the only term without [CO_2], becomes very large compared with the denominator. $\frac{d\,[HCO_3^-]}{d\,[CO_2]}$ approaches ∞ as [CO_2] approaches 0. When [CO_2] is very large $\frac{d\,[HCO_3^-]}{d\,[CO_2]}$ becomes 0. Thus the retention is the more effective the lower the concentration of CO_2 in the absorbing solution. It follows as a practical conclusion that the retention for a given CO_2 pressure in the gas mixture can be improved if the absorption coefficient α can be reduced.

Effect of K_B on retention. Fig. 1 is a plot of the concentration of HCO_3^- in base solutions against the concentration of CO_2 for different values of K_B. The data were calculated from equation 2. It will be seen that when K_B is large (10^{-2} M) most of the base reacts

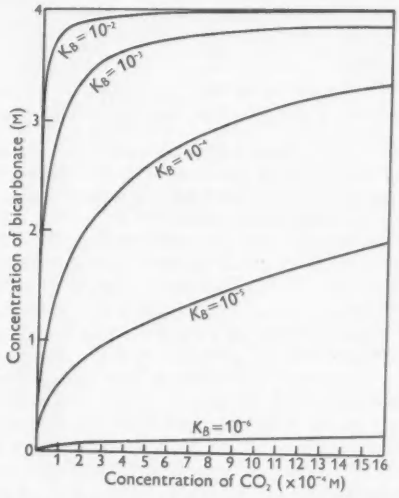

Fig. 1. Concentration of bicarbonate at various CO_2 concentrations in solutions of bases with different dissociation constants (calculated according to equation 2).

with CO_2 according to 1 at very low CO_2 concentrations (below 10^{-4} M). On the other hand, if K_B is very small (10^{-6} M) very little HCO_3^- is formed at the CO_2 concentrations which are of interest, say, between 10^{-3} and 10^{-4} M. There must be a value for K_B at which, for a given CO_2 concentration, the retention will be maximal, i.e. the slope of the plot of [HCO_3^-] against [CO_2] steepest. The K_B value giving maximum retention at a given CO_2 concentration can be calculated by differentiating $\frac{d\,[HCO_3^-]}{d\,[CO_2]}$ with respect to K_B and equating to zero. This leads to the following equation:

$$K_B^{max.} = \frac{2K_W\,c\,(\sqrt{2}-1)}{[CO_2]\,K_{H_2CO_3}}, \quad (4)$$

where $K_B^{max.}$ is the dissociation constant giving maximum retention at the CO_2 concentration [CO_2]. With the help of this equation K_B values giving maximum retention can be calculated for each CO_2 concentration. It follows from equation 4 that $K_B^{max.}$ is directly proportional to the concentration of the retaining base (c) and inversely proportional to the concentration of CO_2. A few examples are

shown in Table 1. It is especially important to use a favourable K_B value at the higher concentrations of CO_2.

Table 1. *Values for* $K_B^{max.}$ *calculated from equation* 4

($K_{H_2CO_3}=10^{-6}$; $K_W=10^{-14}$; all concentrations M)

CO_2	c	$K_B^{max.}$
10^{-4}	4	$3{\cdot}32\times10^{-4}$
10^{-3}	4	$3{\cdot}32\times10^{-5}$
10^{-3}	2	$1{\cdot}66\times10^{-5}$
10^{-3}	1	$0{\cdot}83\times10^{-5}$

In Fig. 2 the retention $\left(\frac{d\,[HCO_3^-]}{d\,[CO_2]}\right)$ is plotted against $-\log K_B$ for various concentrations of base and for a mixture of two bases. The curves show that the peak giving maximum retention is fairly sharp at higher concentrations of base.

Retention in mixtures of bases. A relatively simple case which may be considered is the mixture of an amine with a much stronger base, e.g. sodium hydroxide. In this case CO_2 will react at very low pressures until virtually all the strong base is converted into the bicarbonate. On the assumption that the strong base reacts in fact quantitatively with CO_2 to form bicarbonate before the retaining amine reacts with CO_2, and that the simplifying premises made before are valid, the bicarbonate concentration in the mixture can be calculated from the following equation:

$$[HCO_3^-]=\sqrt{\left\{\frac{(c+b)\,K_BK_{H_2CO_3}[CO_2]}{K_W}+\left(b-\frac{K_BK_{H_2CO_3}[CO_2]}{K_W}\right)^2\right\}}+b-\frac{K_BK_{H_2CO_3}[CO_2]}{K_W}, \qquad (5)$$

where b is the concentration of a monoacidic strong base. Differentiation of equation 5 with respect to $[CO_2]$ gives:

$$\frac{d\,[HCO_3^-]}{d\,[CO_2]}=\frac{\dfrac{(c+b)\,K_BK_{H_2CO_3}}{K_W}-\dfrac{b\,K_BK_{H_2CO_3}}{2K_W}+\left(\dfrac{K_BK_{H_2CO_3}}{K_W}\right)^2\dfrac{[CO_2]}{2}}{2\sqrt{\left\{\dfrac{(c+b)\,K_BK_{H_2CO_3}[CO_2]}{K_W}+\left(b-\dfrac{K_BK_{H_2CO_3}[CO_2]}{K_W}\right)^2\right\}}}-\frac{K_BK_{H_2CO_3}}{2K_W}.$$

This equation is applicable, for example, to (dilute) solutions containing, as does Pardee's (1949) reagent, sodium bicarbonate in addition to diethanolamine. The theoretical effect of the addition of bicarbonate as calculated by this formula is seen in Fig. 2. Addition of a strong base increases the retention and raises the value of $K_B^{max.}$. The increase in retention is smaller than that brought about by an equivalent increase of the concentration of the weak base.

EXPERIMENTAL

Autoxidation of diethanolamine

Solutions of diethanolamine were shaken in air or in O_2 in manometer vessels at 25 or 40°. Measurable quantities of O_2 were always absorbed, but the rates showed considerable variations under apparently identical conditions. For example, at 40° 1 ml. of 4M-diethanolamine, in equilibrium with 5 % CO_2 in O_2, absorbed between 15 and 100 μl. of O_2 per hr. The rate usually increased with the time of incubation. Solutions made from freshly recrystallized diethanolamine showed a very low uptake during the first 30 min., but the rate gradually rose to values similar to those with the commercial material. Addition of *sec.*-octanol increased the rate of oxidation. Among the many substances tested for inhibitory activity, dithizone (*sym.* diphenylthiocarbazone) and thiourea were found to be most effective. Examples of observations made at 40° are given in Table 2. At 25° the rate of oxidation was about 12 % of that at 40°.

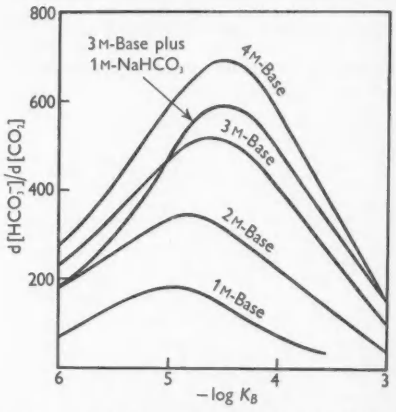

Fig. 2. Dependence of retention d $[HCO_3^-]$/d $[CO_2]$ on K_B for various concentrations of base (calculated according to equation 3; $[CO_2]=10^{-3}$; $K_{H_2CO_3}=10^{-6}$; $K_W=10^{-14}$).

At 40° dithizone (0·02 %) abolished the autoxidation almost completely for 1 or 2 hr., but on prolonged incubation at 40°, or on storage for several days at room temperature, the inhibitory effect gradually disappeared. This resulted in a gradually

Table 2. *Autoxidation of diethanolamine*

(3 ml. 4 M-diethanolamine in equilibrium with 5% CO_2 in O_2; 40°.)

Additions	0	0·02% Dithizone	30 mg. *sec*-Octanol	30 mg. *sec*-Octanol + 0·02% dithizone	0·1% Thiourea	0·1% Thiourea + 30 mg. *sec*-octanol
	O_2 absorbed after equilibration period of 1 hr. (μl.)					
First 20 min.	15	2	54	2	7	7
Second 20 min.	23	2	61	2	8	7
Third 20 min.	37	3	77	3	7	7

rising rate of O_2 absorption. With 0·1% thiourea the initial rate of autoxidation was a little higher than with dithizone (Table 2), but the rate remained steady for more than 24 hr. at 40° or for several weeks at room temperature. Thiourea is therefore the more useful general antioxidant.

Experimental test of equation 2

Manometric flasks of the Dickens & Šimer (1931) type were filled as follows:

Inner vessel	0·0100 M-Triethanolamine, 3 ml.
Outer vessel	Na_2CO_3 solution of various strengths, 2 or 3 ml.
Side arms (both)	0·5 M-H_2SO_4, 0·5 ml.
Gas	N_2

The triethanolamine used was freshly purified according to Germann & Knight (1935). The vessels were shaken at 25·0° until the manometric readings were constant. The acid was then added to the carbonate in the outer vessel and the manometers were shaken until the readings indicated that equilibrium between gas phase and solution had been established. The increase in pressure—the CO_2 pressure over the triethanolamine solution—was read and the acid was then added to the triethanolamine solution to measure the concentration of bound CO_2 in this solution.

In Fig. 3 the concentration of bicarbonate in the amine solution is plotted against the CO_2 pressure. The curve is that calculated on the basis of equation 2, using for $K_B^{25°}$ the value $5{\cdot}97 \times 10^{-7}$ (Hall & Sprinkle, 1932), for $K_W^{25°}$ $1{\cdot}01 \times 10^{-14}$ (Harned & Owen, 1944), for $K_{H_2CO_3}^{25°}$ $5{\cdot}00 \times 10^{-7}$ (Roughton, 1943) and for $\alpha_{CO_2}^{25°}$ 0·760. The crosses in the figure represent the measured data. The agreement between theory and measurement is very satisfactory.

Another test of the theory is as follows. According to the theory it is to be expected that

$$\frac{[CO_2]\,[\text{Undissociated base}]}{[HCO_3^-]\,[\text{Base cation}]} = \frac{K_W}{K_{H_2CO_3} K_B}.$$

As the concentration of the base cation is virtually equal to that of bicarbonate and the concentration of undissociated base equals $[C - HCO_3^-]$ where C is the total concentration of triethanolamine, the above equation becomes

$$\frac{[CO_2]\,[C - HCO_3^-]}{[HCO_3^-]^2} = \frac{K_W}{K_{H_2CO_3} K_B} = K'.$$

Data for K' are given in the last column of Table 3. The values obtained are constant. The experimental error is expected to be relatively high at low CO_2 pressures (because a small error in the manometer reading causes a large percentage error in p_{CO_2}) and at high bicarbonate concentrations (because a small percentage error in $[HCO_3^-]$ causes a large error in $[C - HCO_3^-]$). If the data below 50 mm. CO_2 and above 0·008 M-HCO_3^- are omitted for the calculation of the average, the average value of K' is $3{\cdot}23 \times 10^{-2}$; the average of all values is $3{\cdot}16 \times 10^{-2}$. The value calculated from the data for the constants quoted above from the literature is $3{\cdot}39 \times 10^{-2}$.

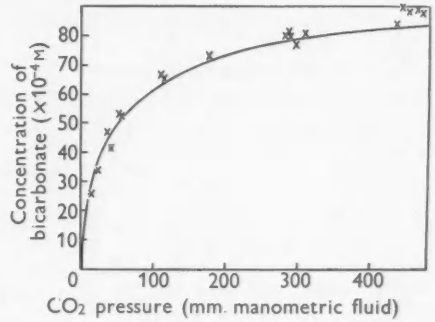

Fig. 3. Concentration of bicarbonate in 0·0100 M-triethanolamine solutions at different CO_2 pressures. (Experimental test of equation 2. The curve is calculated from equation 2; the crosses are the data measured. For details of procedure see text, p. 353).

It is evident that if K_{CO_2} and K_W are known the dissociation constant of a base can be ascertained by this procedure. The value obtained in this way for triethanolamine ($6{\cdot}25 \times 10^{-7}$) agrees reasonably well with that reported by Hall & Sprinkle (1932) ($5{\cdot}97 \times 10^{-7}$).

Table 3. *Concentration of bicarbonate in triethanolamine solutions at various* CO_2 *pressures*

(0·0100 M-triethanolamine; 25°; initial gas N_2; $\alpha^{25°}_{CO_2}=0·76$; for significance of K' see text, p. 353.)

CO_2 pressure (mm. manometric fluid)	Concentration of CO_2 in solution ($\times 10^{-3}$ M)	HCO_3^- found in 3 ml. (μl.)	Concentration of HCO_3^- ($\times 10^{-2}$ M)	K'
12	0·0410	176	0·262	0·0440
13·5	0·0461	174	0·258	0·0507
22	0·075	232	0·344	0·0446
37	0·126	314	0·466	0·0306
40·5	0·138	286	0·425	0·0436
55	0·188	356	0·529	0·0313
56	0·191	354	0·526	0·0327
111	0·380	446	0·664	0·0287
112·5	0·384	441	0·656	0·0302
175	0·597	493	0·733	0·0295
282	0·962	541	0·805	0·0286
287	0·980	545	0·810	0·0280
288	0·983	529	0·785	0·0337
296	1·010	519	0·770	0·0385
309	1·056	545	0·810	0·0301
435	1·485	566	0·844	0·0327
443	1·515	608	0·905	0·0175
455	1·555	595	0·885	0·0227
464	1·585	601	0·895	0·0208
471	1·610	591	0·880	0·0247
726	2·48	606	0·902	0·0298

Measurement of retention

A known amount of CO_2 was liberated in a manometric flask containing the retaining fluid and the amount of CO_2 not absorbed by the retaining fluid was measured. Dickens & Šimer (1931) flasks, provided with valve stoppers, were filled as follows:

Inner compartment	Retaining solution to be tested, 0·5 ml.
Outer compartment	0·010 M-$NaHCO_3$ solution, 2 ml.
Side arm of outer compartment	3 M-H_2SO_4, 0·25 ml.

Before the amine solution was measured into the flask a rapid stream of CO_2 was passed through it for about 10 min. to neutralize about half the amine with CO_2. This expedited the subsequent equilibration of the retaining solution with the gas mixture.

The manometers with their flasks were placed into the water bath and a gas mixture containing CO_2 in N_2, saturated with water by passage through a wash bottle immersed in the water bath, was sent through the vessels whilst they were shaken at a rapid rate in the water bath. This was continued until the gas equilibrium was reached, usually for 30–60 min. To test the equilibrium the tap and the valve stopper were closed; on further shaking the pressure changes should not exceed a few mm. N_2 rather than O_2 was used to avoid complications from autoxidation.

This procedure was adopted after many preliminary trials. When larger quantities of amine solution were used the time required for equilibration was greatly prolonged.

When equilibrium had been established and the pressure read, the acid in the side arm was mixed with the bicarbonate solution and the manometers were again shaken until constancy of pressure was attained. If a = the quantity of CO_2 liberated from bicarbonate in the outer compartment (in μl.); h = the CO_2 pressure remaining when absorption by the amine solution was completed (in mm. manometric fluid); b = the quantity of CO_2 not absorbed by the base in the inner vessel (in μl.), and v_F = the volume of the amine solution (in ml.) the average retention $\left(=\dfrac{\Delta\ [\text{retained } CO_2]}{\Delta p\ CO_2\ v_F}\right)$ for the pressure increase h was $(a-b)\ hv_F$.

Over two hundred measurements were made in the search for the best CO_2 buffers for varying CO_2 pressures at 25 and 40°. The number of suitable bases is limited by three requirements: the solubility must be high enough to allow the preparation of concentrated solutions; the substance must not be volatile lest it should distil into other compartments of the manometric vessel (this excludes ammonia and many alkyl amines); and K_B must be of the order of 10^{-5}. The amines tested included monoethanolamine, diethanolamine, triethanolamine, mono*iso*propanolamine, di*iso*propanolamine, tri*iso*propanolamine, ethylenediamine, diethylenetriamine, triethylenetetramine and 1:3-diaminopropanol. In addition to pure solutions of bases, mixtures of bases were tested as well as the solutions recommended by Pardee (1949), prepared by mixing diethanolamine, powdered potassium bicarbonate and hydrochloric acid.

Table 4. *Retention of CO_2 by amine solutions*

(For procedure see text, p. 354; the solutions according to Pardee (1949) varied with the CO_2 pressure and were prepared for use at 40 and 25° according to the directions given by Pardee for 38°.)

Temp. (°)	Initial CO_2 content of gas mixture per cent (v/v)	Solutions tested	Retention
40	0·94	4 M-Diethanolamine	50
		5 M-Diethanolamine	45
		4 M-Diethanolamine, 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	49
		Pardee's solution	51
40	1·98	4 M-Diethanolamine	28
		5 M-Diethanolamine	31
		4 M-Diethanolamine, 1 M-monoethanolamine	31
		4 M-Diethanolamine; 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	37
		Pardee's solution	41
40	3·09	4 M-Diethanolamine	16·7
		5 M-Diethanolamine	23·3
		4 M-Diethanolamine, 1 M-monoethanolamine	13·0
		4 M-Diethanolamine; 4 % (w/v) $KHCO_3$; 12 % (w/v) KCl	22·4
		Pardee's solution	22·0
40	3·93	4 M-Diethanolamine	12·3
		5 M-Diethanolamine	12·3
		4 M-Diethanolamine, 1 M-monoethanolamine	10·5
		4 M-Diethanolamine; 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	11·3
40	5·74	3·75 M-Diethanolamine	10·2
		4 M-Diethanolamine	10·5
		4·5 M-Diethanolamine	9·5
		4 M-Diethanolamine, 1 M-monoethanolamine	11·4
25	0·94	4 M-Diethanolamine	29
		4 M-Diethanolamine; 12 % (w/v) $KHCO_3$; 6 % (w/v) KCl	44
		Pardee's solution	140
25	1·98	4 M-Diethanolamine	25·6
		5 M-Diethanolamine	19·6
		4 M-Diethanolamine, 1 M-monoethanolamine	17·8
		Pardee's solution	25·3
25	3·09	4 M-Diethanolamine	16·6
		5 M-Diethanolamine	13·8
		4 M-Diethanolamine, 1 M-monoethanolamine	13·0
		Pardee's solution	17·3
25	3·93	4 M-Diethanolamine	10·3
		5 M-Diethanolamine	9·2
		4 M-Diethanolamine, 1 M-monoethanolamine	8·2
		Pardee's solution	6·6
25	5·74	4 M-Diethanolamine	9·5

Neutral salts were added to the amine solutions in the expectation that this might increase retention by reducing the solubility of CO_2 in the solution, the expected 'salting out' effect being the greater the smaller the ion and the greater the charge (see Glasstone, 1941). As the salt employed must be very soluble and not form sparingly soluble compounds with the amine or with bicarbonate, the range of promising substances was limited. Several halides and sulphates of the alkali metals were tested. Some effects in the expected direction were noted (see below), but no substance was found to be generally effective.

Only a few selected results are given in Table 4. Omitted are data showing low retention as these are not of interest in connexion with the object of this paper. Diethanolamine in various combinations gave throughout higher retention values than other substances tested. The main points emerging from the data are the following:

(1) In general the retention for the same solution, e.g. 4 or 5 M-diethanolamine, fell as the CO_2 pressure rose. At 40° retention was greater than at 25°, as is theoretically expected because of the fall in α with rising temperature.

(2) At 40° the highest retention values were given under most conditions by 4 or 5 M-diethanolamine. The solutions recommended by Pardee (1949) gave similar values with 1 and 3 % CO_2 and somewhat higher retention with 2 % CO_2. Addition of potassium bicarbonate or potassium chloride increased retention with 2 and 3 % CO_2, but not with 1 or 4 %.

(3) At 25° exceptionally high retention was observed with Pardee's solution with 1 % CO_2. At this gas pressure the addition of potassium bicarbonate and potassium chloride to 4 M-diethanolamine increased retention considerably. An unexplained discrepancy was observed between the solution of Pardee (prepared from diethanolamine, potassium bicarbonate and potassium chloride), which gave a value of 140, and a solution expected to have the same composition prepared from diethanolamine, potassium bicarbonate, potassium chloride and CO_2, which gave 44.

(4) 4 M-Diethanolamine is the best reagent for most conditions, the only major exception being for 1 % CO_2 at 25°. In the majority of cases 4 M-diethanolamine is as effective as are Pardee's mixtures (which contain about 3·8 M-diethanolamine). No solutions appreciably more effective than those recommended by Pardee were found.

Procedure for the use of CO_2 buffers

Choice of experimental arrangements. The data in Table 4 may be used for calculating the effectiveness of retention under different conditions, the percentage retention being

$$\frac{v_F R}{v_F R + k_{CO_2}} \times 100,$$

where v_F is the volume of the retaining fluid, k_{CO_2} the vessel constant for CO_2 and R the retention given in the last column of Table 4. For Pardee's solutions as used by Pardee ($v_F = 0{\cdot}6$; $k_{CO_2} = 1{\cdot}3$) the following values result: 1 % CO_2, 96 % retention; 2 % CO_2, 94 % retention; 3 % CO_2, 83 % retention. As Pardee has already pointed out, his conditions may be regarded as satisfactory for CO_2 pressures up to 3 %.

To increase retention with a given value for the retaining capacity of the solution, it is necessary to reduce k_{CO_2} or to increase v_F. The effects of variation in k_{CO_2} and in the volume v_F may be seen from Table 5, where data are shown for the volume required to give 90 and 95 % retention for two values of k_{CO_2}. v_F was calculated according to the formula

$$v_F = \frac{k_{CO_2}}{R} \times \frac{a}{100 - a},$$

where a is the percentage retention. It will be seen that, except with 1 % CO_2, larger volumes of retaining fluid are required than can be used in the centre well of the conventional conical manometer flask. On the other hand, the vessels designed by Dickens & Simer (1931), already recommended by Pardee, or those described by Warburg, Kubowitz & Christian (1931), are suitable; either the centre compartment or the outer ring can be filled with several ml. of fluid. In these vessels sufficiently large volumes of retaining fluid can be used to make 98 % retention practicable at 1 and 2 % CO_2, but not at higher CO_2 pressures. It is clear that 'complete' absorption of CO_2 is not always obtainable, but in many types of experiment 90 or 95 % retention may be satisfactory.

In order to test whether diethanolamine may distil from one manometer compartment into another under the experimental conditions, Dickens-Šimer vessels containing 3 ml. of 4 M-diethanolamine (prepared from 'diethanolamine technical' of British Drug Houses Ltd) in one compartment and 3 ml. of 1 % potassium dihydrogen phosphate solution in the other, were rapidly shaken for 4·5 hr. at 40°. The gas space contained 3 % CO_2 in O_2. After incubation the phosphate solution was wet ashed with sulphuric acid. No measurable quantities of ammonia, i.e. less than 0·005 mg. nitrogen, were found. The danger of contamination with diethanolamine by distillation is therefore negligible.

Preparation of CO_2 buffers. It is essential to equilibrate the buffer solution (before it is pipetted into the vessel) with the same CO_2 mixture as is used in the experiment. The equilibration has to be carried out at the temperature at which the solution is finally used, but a stock of equilibrated buffer may be kept for several weeks at room temperature if stored in a stoppered cylinder.

Table 5. *Volume of retaining fluid (4 M-diethanolamine) required for 90 and 95 % absorption of CO_2*

CO_2 concentration (%)	Retention	Temp. (°)	Volume required (ml.) $k_{CO_2}=1{\cdot}5$ 90% absorption	$k_{CO_2}=1{\cdot}5$ 95% absorption	$k_{CO_2}=2{\cdot}5$ 90% absorption	$k_{CO_2}=2{\cdot}5$ 95% absorption
1	50	40	0·27	0·57	0·45	0·95
2	28	40	0·48	1·02	0·50	1·79
3	17	40	0·80	1·68	1·32	2·80
4	12	40	1·10	2·33	1·88	3·95
1	29	25	0·47	0·98	0·78	1·64
2	26	25	0·52	1·10	0·87	1·83
3	17	25	0·80	1·68	1·32	2·80
4	10	25	1·35	2·85	2·35	4·75

Stock solutions of 4 M-diethanolamine buffer are prepared by passing first a rapid stream of 100 % CO_2 through one-third of the desired quantity of solution until saturation is obtained. This is tested with phenolphthalein (pH ~ 8) and can be achieved in 30 min. if a sintered glass distributor of the type used for aerating aquaria is used. The remaining two-thirds of the untreated solution are then added and the gas mixture to be used in the manometer is passed through the solution at the appropriate temperature for 1 or 2 hr., again through a sintered glass distributor. Whether equilibrium has been reached may be tested as follows: the solution (3 or 4 ml.), together with a few glass beads of 4–5 mm. diameter, is placed in a manometer provided with a valve stopper (Warburg & Kubowitz, 1929) and the gas mixture is passed through the vessel whilst it is being shaken in the bath. The further procedure is as described on p. 354.

Diethanolamine 'technical' was found to be as satisfactory as a recrystallized sample, provided that thiourea (0·1 %) was added. Thiourea should be dissolved before the solutions are equilibrated with the gas mixture.

If the solutions of Pardee are used, thiourea (0·1 %) should also be added and equilibrating and testing should be carried out as here described except for the omission of the primary treatment with 100 % CO_2. This procedure is recommended because the CO_2 pressures stated by Pardee are only approximately correct.

Controls. A manometer vessel without the metabolically active material but containing the buffer solution and the same gas mixture is required in each series of experiments. This vessel may also serve as the thermobarometer.

Test of the method

Anaerobic fermentation of baker's yeast. To test whether the absorption of CO_2 in the presence of CO_2 buffers is of the expected order, yeast suspensions steadily producing CO_2 were placed in manometer vessels, with and without a CO_2 buffer. Dickens-Šimer flasks were used. The outer compartment contained 2 ml. of yeast suspension in 0·05 M-potassium dihydrogen phosphate solution and the side arm attached to it 1 ml. of 0·1 M-glucose solution. The CO_2 buffer (3 ml. of 4 M-diethanolamine) and five glass beads were placed in the centre compartment. The side arm attached to this compartment contained yellow phosphorus; the gas mixture consisted of 3·6 % CO_2 and 96·4 % N_2. The bath temperature was 25°. The vessels were gassed in the bath for about 20 min. After readings had shown that gas equilibrium was established, the glucose was mixed with the yeast cells. The gas evolution observed after mixing is shown in Table 6. It will be seen that the absorption of CO_2 was less than expected during the first two 10 min. periods, but afterwards it was of the expected order. In other words, after about 20 min. a steady state developed where the CO_2 pressure was about 15 and 30 mm. respectively above the equilibrium pressure. Once the steady state was attained, absorption was according to the theory.

In further experiments of this type the level of excess CO_2 pressure in the steady state was found to vary with the shape and size of the vessel, the rate of shaking and especially the rate of CO_2 production. In the experiment shown in Table 6 where the two vessels containing CO_2 buffer were of identical size ($k_{CO_2} = 2·36$) the quantity of non-absorbed CO_2 was about proportional to the rate of CO_2 production. It made no difference to the efficiency of the CO_2 buffer if it was placed in the outer compartment instead of the centre. From the practical point of view it is important that in all experiments the steady state is established within 20–40 min. If a continuous reaction is being studied most of this time would fall within the usual period of gas equilibration.

Respiration of Escherichia coli. In this experiment the rates of O_2 consumption of a bacterial suspension as measured by the new method and by the standard method (sodium hydroxide in centre well) were compared. *Esch. coli* was grown on an agar medium containing 1 % casein digest, 0·2 % yeast extract and 2 % glycerol. The cells were washed with water and suspended in 0·01 M-phosphate buffer, pH 6·8.

Table 6. *Effectiveness of* CO_2 *buffer. Absorption of* CO_2 *produced by fermenting yeast cells*

(The weight of yeast refers to fresh weight. Rate of shaking: 100 oscillations/min.; amplitude 5 cm.; $k_{CO_2} = 2·36$. For further particulars see text, above)

	5 mg. yeast/vessel			10 mg. yeast/vessel		
	CO_2 evolved			CO_2 evolved		
Time period (min. after addition of glucose)	Without CO_2 buffer (μl.)	With CO_2 buffer (μl.)	CO_2 absorbed (%)	Without CO_2 buffer (μl.)	With CO_2 buffer (μl.)	CO_2 absorbed (%)
0–10	29	24	17	53	38	28
10–20	40	10	75	90	24	73
20–40	91	4	95	198	23	88
40–60	87	6	93	196	10	95
60–120	234	8	97	453	18	96

Table 7. *Respiration of* Escherichia coli *in the presence and absence of* CO_2

(Duplicate measurements; readings began 20 min. after addition of substrate; for further particulars see text, p. 357.)

Time of observation	Substrate (final concn. 0·033 M)	Q_{O_2}: 3 % CO_2 in O_2; CO_2 buffer	Q_{O_2}: O_2; 2 N-NaOH
First 40 min.	Glucose	32·6, 36·7	31·1, 30·7
Second 40 min.	Glucose	36·7, 42·4	34·1, 33·0
First 40 min.	Sodium fumarate	63·4, 63·0	66·0, 65·7
Second 40 min.	Sodium fumarate	69·2, 65·5	63·7, 62·6

Table 8. *Respiration of Fleischmann's yeast. Effect of metabolic rate on efficiency of* CO_2 *buffer*

(Temp. 25°. The data for weights refer to fresh weight. Rate of shaking: 90 oscillations/min.; amplitude 5 cm.; the data for O_2 absorption in the presence of CO_2 buffers are not corrected for incomplete absorption. With the higher yeast cell concentrations the pressure changes exceeded the scale length. When the bottom of the scale was approached the manometer level was re-adjusted by injection of O_2 into the vessel. Readings were resumed after 5 min. and the O_2 uptake for this interval was calculated by interpolation.)

Period of observation (min. after addition of substrate)	O_2 absorbed (μl.): 3·6 % CO_2 in O_2; CO_2 buffer: 64 mg. cells	32 mg. cells	16 mg. cells	8 mg. cells	O_2, NaOH: 64 mg. cells	32 mg. cells	16 mg. cells	8 mg. cells
0–10	32	22	14	7	—	50	32	9
10–20	152	80	42	19	214	104	47	18
20–30	233	115	61	29	285	135	69	32
30–50	540	266	134	64	687	329	161	81
50–70	624	319	160	80	689	338	177	86
70–90	627	313	163	79	707	338	160	81

The manometric arrangement was the same as in the previous experiment, except that the yeast suspension was replaced by 2 ml. of bacterial suspension containing 4 mg. (dry weight) of cells. The bath temperature was 40°. Dickens-Šimer cups were also used for the standard method, the CO_2 buffer being replaced by the same volume of 2 N-sodium hydroxide, and 3 % CO_2 in O_2 by O_2. The results are shown in Table 7. No correction was made for incomplete (about 96 %) absorption of CO_2. It will be seen that the presence of 3 % CO_2 had no major effect on the rate of respiration. Owing to incomplete CO_2 absorption the rates in the presence of CO_2 buffer would be expected to be slightly lower; in fact they are slightly higher than in the presence of sodium hydroxide.

Respiration of baker's yeast. Graded quantities of Fleischmann's yeast were shaken in 3 ml. of 0·07 % potassium dihydrogen phosphate solution containing 0·033 M-glucose. In one series the gas was O_2; CO_2 was absorbed by 3 ml. 2 M-sodium hydroxide placed in the centre compartment of a Dickens-Šimer vessel. In the other, the gas was 3·6 % CO_2 in O_2; CO_2 was absorbed by 3 ml. of 4 M-diethanolamine buffer. The general experimental arrangements were the same as in the previous two experiments. The substrate was added to the yeast suspension from the side arm after gas equilibrium had been established. The results are shown in Table 8. In the first 10 min. after the addition of the substrate diethanolamine was much less efficient than sodium hydroxide as a CO_2 absorbent at all four yeast concentrations tested. From the second 10 min. period onwards the O_2 uptakes observed with diethanolamine and with sodium hydroxide were about the same at the two lower yeast concentrations. At the next higher yeast concentrations the rates of O_2 absorption became approximately equal 50 min. after the addition of the substrate (taking into consideration a correction factor for incomplete absorption). At the highest yeast concentration, where the rate of CO_2 production rose to over 30 μl./min., the absorption of CO_2 was below the expected value throughout the period of observation. It follows that except for a short initial period diethanolamine buffer is as efficient an absorbent for CO_2 as sodium hydroxide as long as the rate of CO_2 production does not exceed, say, 10 μl./min.

DISCUSSION

The study of the theory and the measurement of retention confirms the practicability of the principle introduced by Pardee. It must be stressed that careful equilibration of the buffer with the gas mixture and an adequate rate of shaking are essential in the use of the method; further that trials must be carried out for each set of experiments to test whether the absorption of CO_2 by the buffer is

adequate at the rate of CO_2 production at which it is used. In general it is advisable to adjust the quantity of material in the flasks so that the rate of CO_2 production does not exceed 6–10 μl./min., the upper limit being determined by the design of the vessels and the rate of shaking.

For the measurement of gas exchanges in the presence of CO_2 Pardee's method is simpler and more accurate than the older methods, e.g. the 'two-vessel method' of Warburg (1924). The new method, however, is applicable only to CO_2 pressures below, say, 6%, whilst the 'two-vessel method' has no limitations of this kind.

As the presence of CO_2 may influence the metabolic activity of cells and tissues, the absorption of CO_2 by strong alkali, as commonly practised in the measurement of the O_2 uptake, is often undesirable and in these cases Pardee's method will be of great value. The measurements reported in this paper show that 'complete', say over 98%, absorption is difficult to obtain except at CO_2 pressures below 2%. But even incomplete absorption of CO_2, although it might result in inaccuracies of the measurement of the O_2 uptake, is not necessarily a serious drawback as the magnitude of the error can be assessed. Moreover, in many investigations the measurement of the oxygen consumption is a side issue serving as a measure of the intactness of the energy-giving systems, the main aim being the study of other cell activities such as synthetic processes, absorption or secretion. These processes are often affected by the presence of CO_2 and bicarbonate.

SUMMARY

1. A mathematical theory is given of Pardee's (1949) 'CO_2 buffers' which are designed to maintain an approximately constant carbon dioxide pressure in manometer vessels in which CO_2 is produced or absorbed. The theory is based on simplifying assumptions and indicates how the dissociation constants and the concentration of the base, and the CO_2 pressure, affect the efficiency of the buffer.

2. The buffering capacity or 'retention' of various bases has been measured at 25 and 40° for CO_2 pressures between 1 and 5·7%. Diethanolamine solutions were found to be the best general CO_2 buffers. Their effectiveness may be increased by various additions.

3. Diethanolamine solutions absorb measurable quantities of oxygen. The autoxidation is inhibited by dithizone or thiourea. Controls are required in each experimental series in order to allow for the O_2 uptake of the CO_2 buffer.

4. Absorption of CO_2 exceeding 95% is obtainable at low CO_2 concentrations (1 or 2%) when traditional Warburg manometer vessels are used. With the vessels of Dickens & Šimer, allowing the use of several ml. of CO_2 buffer, absorption over 95% is possible for gas pressures up to 4%.

5. Particulars concerning the preparation and use of CO_2 buffers and examples showing their efficiency are given.

The author is indebted to Dr E. E. Crane for help with the mathematical aspects of this paper, and to Mr R. Hems for technical assistance.

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The Effect of Alcohols on Cholinesterase

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Interest in the mode of action of narcotics has induced a number of workers to study the effect of this group of compounds on cholinesterase, on account of the significance of the latter in the transmission of nervous impulses.

The effect of alcohols has been investigated in some detail by Plattner & Galehr (1928), Bernheim & Bernheim (1936) and Mik'helson (1941, 1943, 1946), who have all found that they cause inhibition of the enzyme. Ettinger, Brown & Megill (1941) have also brought forward evidence in support of this. Heim & Fahr (1940), however, have reported that ethyl and amyl alcohols in low concentrations activate cholinesterase, though inhibition is observed at higher concentrations. Genuit & Labenz (1941) completely failed to substantiate these findings as far as the activation was concerned.

In the course of other work we have repeatedly observed that *n*-propanol and *iso*propanol activate cholinesterase.

The object of the present investigation has been to study the effect of alcohols on cholinesterase in the hope of resolving these apparent contradictions.

EXPERIMENTAL

Cholinesterase activity was determined by a modification of the method of Ammon (1933), i.e. by the Warburg manometric technique. The saline introduced by Krebs & Henseleit (1932) was employed. It was equilibrated with 5% CO_2, 95% N_2 before use, in addition to the final equilibration of the filled flasks which was carried out in the thermostat at 38°. In the earlier part of the work, the enzyme and substrate were mixed, and 15 min. was then allowed for temperature equilibration before reading. In the later experiments, in which the activity at low substrate concentrations was studied, the linear period of CO_2 output was inevitably reduced (see Figs. 8 and 11); to obtain satisfactory results, it was necessary to allow only the minimum effective equilibration period after mixing and to read more frequently. In practice readings were taken every 5 min.

An attempt was made to measure the output during the first 5 min. by reading the manometer before mixing. This led to the observation that the addition of the alcohol caused in the first 5 min. an immediate evolution of gas which might be several times greater than the subsequent 5 min. output. This initial evolution has been shown to occur in the absence of both enzyme and substrate.

The output rises steeply with butanol concentration, but is independent of the presence of substrate (Fig. 1). The latter point is of considerable practical significance, since it means that the substrate concentration present during the period when useful measurements can be taken has not been affected by the initial output. The gas evolution may be due to the influence of the alcohol on either the pK of $NaHCO_2$ or the solubility of CO_2, for if the alcohol and $NaHCO_3$ are mixed in the well of the flask before equilibrating with the gas mixture and the enzyme is placed in the side arm, there is no abnormal gas evolution after mixing.

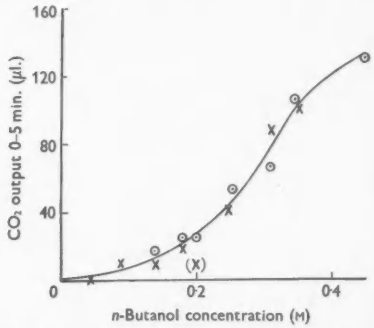

Fig. 1. Effect of *n*-butanol concentration on the CO_2 output during the first 5 min. following the mixing of butanol and buffer (enzyme absent). ×——×, substrate (0·018 M-acetylcholine) present; ⊙——⊙, substrate absent.

Enzyme preparations. The greater part of the work has been carried out on a homogenate of rat brain. Male white rats were killed, exsanguinated and the brain removed rapidly, washed in Krebs-Henseleit saline, blotted and homogenized in the saline (18·0 ml. of saline to 1 g. of tissue). The homogenizer used was similar to that of Folley & Watson (1948). The brain was homogenized for 10–15 min. at 12,000 rev./min.

The human erythrocyte preparation used consisted of stroma from cells which had been washed several times to remove serum. They were haemolysed in 6 vol. of water, washed with several changes of water until the supernatant was colourless and then freeze dried. The preparation consisted of a 0·2% suspension in Krebs-Henseleit saline. Horse serum was diluted 1 in 5 with this saline before use. In each case 0·5 ml. of enzyme was employed in a total volume of 3·0 ml.

Substrates. Acetylcholine, acetyl β-methylcholine (Amechol: Savory and Moore) and benzoylcholine have all been used. The substrate solutions were made up freshly for each experiment to minimize falling-off in concentration prior to the experiment due to non-enzymic hydrolysis. For the same reason, they were dissolved in distilled water and not in buffer solution.

Alcohols. The alcohols used were the purest grade available commercially and the *n*-butanol, in particular, was of A.R. quality. We are indebted to British Drug Houses Ltd. for providing the latter before it actually came on the market.

Controls. The possibility that the alcohol would affect the rate of non-enzymic hydrolysis has been checked. The effect of *n*-butanol was negligible in the case of all three substrates.

A control experiment was also made in which the substrate only was omitted. This was to investigate the possibility that the apparent activation might be merely an oxidation of the alcohol by other enzymes in the tissue. The output for the 20 min. period between 5 and 25 min. after mixing was found to be:

(i) In the absence of butanol, $3{\cdot}9 \pm 2{\cdot}6$ (S.D.) mm.^{3} CO_2.

(ii) In the presence of butanol, $4{\cdot}7 \pm 1{\cdot}1$ (S.D.) mm.^{3} CO_2.

There is obviously no significant difference between the two values (Student's $t = 0{\cdot}77$), the mean of which is 4·3. The output falls to less than half this value for the ensuing 30 min.

In the experiments at low substrate concentrations, in which the 5 min. output has been the basis for calculation, a correction of 1·1 has been deducted, but in the earlier long-period experiments this correction, which would have been less, has been neglected. This slight output is assumed to be due to residual metabolism.

RESULTS

The effect of the nature and concentration of aliphatic alcohols on the activity of rat-brain cholinesterase

Straight-chain alcohols. The effect of primary alcohols on the activity of the enzyme is shown in Fig. 2*a*. The activity was determined after the enzyme had been incubated with the alcohol for 60 min. at 38°, the substrate being 0·018 M acetylcholine. The main conclusions to be drawn are: (i) Alcohols containing 3–6 carbon atoms activate the enzyme. The effect is maximal with *n*-butanol, the maximum activation being 75 %. (ii) At concentrations above the optimum required for the production of activation the alcohol brings about an inactivation of the enzyme. This is practically complete at a concentration three times the optimum. (iii) The optimum activating concentration decreases rapidly as the length of the carbon chain increases, being approximately halved for each additional carbon atom.

It should be pointed out that the falling-off in activation observed after the four-carbon alcohol cannot be explained in terms of decreasing solubility, since the six-carbon alcohol, which hardly activates at all, will inhibit completely when its concentration is further increased. The optimum activating concentrations are actually about one-third to one-half of the saturation concentrations (Seidell, 1941).

Branched-chain alcohols. Only two alcohols containing branched chains have been studied, namely *iso*propanol and *iso*butanol. They were tested under the same conditions as the straight-chain alcohols. The general form of the curves relating alcohol concentration and activity has been found to be the same as for the latter (Fig. 2*b*). *iso*Propanol is, however, considerably more active than its primary isomer, being only just less active than *n*-butanol, whereas *iso*butanol is less active than its isomer. In both cases the optimum activating concentration is some 50 % higher than that for the corresponding straight-chain alcohols.

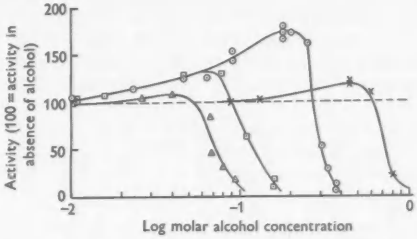

Fig. 2*a*. Effect of different concentrations of primary alcohols on the cholinesterase activity of rat-brain homogenate. *n*-Propanol, ×——×; *n*-butanol, ⊙——⊙; *n*-pentanol, □——□; *n*-hexanol, △——△. Enzyme and alcohol incubated for 60 min. at 38° (pH 7·6) prior to measurement of activity. Substrate 0·018 M-acetylcholine.

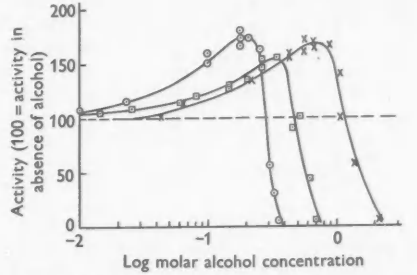

Fig. 2*b*. Effect of different concentrations of secondary alcohols on the cholinesterase activity of rat-brain homogenate. *iso*Propanol, ×——×; *iso*butanol, □——□; *n*-butanol, ⊙——⊙ (reference standard taken from Fig. 2*a*). Enzyme and alcohol incubated for 60 min. at 38° (pH 7·6) prior to measurement of activity. Substrate 0·018 M-acetylcholine.

The consistent pattern of the phenomenon, namely activation rising to a maximum followed by actual inhibition as the alcohol concentration is further increased, suggests that two effects are involved. The inhibition phase is not unexpected, since the concentration required to produce it might easily bring about denaturation of the enzyme. However, there appears to be no obvious explanation of the activation. Since *n*-butanol exhibits the effect to the greatest extent and moreover gives more reproducible results than are obtained with

n-propanol, work designed to throw light on the mechanism of the process has been carried out with the former compound only.

The effect of n-*butanol on brain cholinesterase*

The influence of time and temperature. The period of incubation used in the work described above had been arbitrarily set at 60 min., but as equilibrium seemed to be reached in a much shorter time the technique was altered to eliminate the initial incubation period.

This actually leads to a greater degree of activation at all alcohol concentrations, a maximum of over 100% activation being achieved (Fig. 3). The

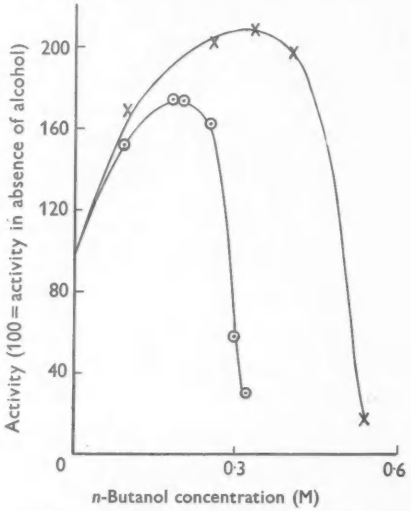

Fig. 3. Influence of incubation time on the effect of *n*-butanol on the activity of cholinesterase (rat-brain homogenate). Substrate 0·018M-acetylcholine. ⊙——⊙, 60 min. incubation at 38° prior to measurement of activity (curve taken from Fig. 2*a*); ×——×, no incubation prior to measurement of activity.

optimum activation concentration is also higher. These findings fit in well with the previous suggestion that two processes are occurring, provided it is assumed that the activation is rapid and the inhibition slow. The upper curve is then a measure of the activation process, and the difference between the two curves a measure of the rate of inactivation.

Both processes are dependent on the alcohol concentration. However, the rate of inactivation appears to increase very rapidly in the neighbourhood of 0·4M so that almost instantaneous inactivation occurs.

The effects of time and temperature on activation and inactivation, shown in Fig. 4, suggest (*a*) that the activation is instantaneous and (*b*), that as the inactivation is very rapid at 38° but practically negligible at 23°, the mechanism may be analogous to denaturation, a process frequently found to have a high temperature coefficient (cf. Stearn, 1949).

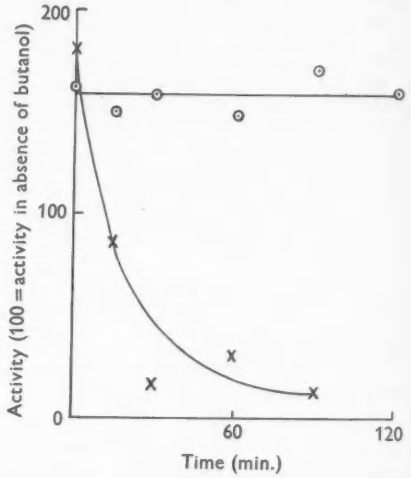

Fig. 4. The influence of temperature on the effect of *n*-butanol on the activity of cholinesterase (rat-brain homogenate). Abscissae: length of incubation prior to measurement of activity. ⊙——⊙, incubated at 23°; ×——×, incubated at 38°.

The reversibility of the action of butanol on brain cholinesterase

The enzyme, activated by the addition of 0·19M-butanol, was dialysed against several changes of Krebs-Henseleit saline. The activation was found to be completely reversible by dialysis (Table 1*a*).

Table 1. *The effect of dialysis on butanol-activated and butanol-inactivated cholinesterase (rat-brain homogenate)*

(Substrate, 0·018M-acetylcholine.)

	Relative cholinesterase activity	
	In absence of butanol	In presence of butanol
(*a*) Activated		
Before dialysis	100	178
After dialysis	103	93
(*b*) Inactivated		
Before dialysis	100	10
After dialysis	104	5

If, however, the enzyme is dialysed after inactivation by prolonged contact with a higher concentration of butanol, there is no sign of reversal of the

inactivation, even after 44 hr. dialysis in which the smell of the alcohol disappeared with the third of five changes of saline.

These results provide additional evidence for the suggestion that two separate mechanisms are involved: a reversible activation process and an irreversible inactivation. Denaturation is to be expected at the concentration at which the latter occurs, hence the inactivation phenomenon has not been examined further.

The effect of altering the enzyme and substrate

The mechanism of activation was examined by studying the influence of the enzyme and the substrate. The following combinations of enzyme and substrate were employed: (*a*) rat-brain homogenate and acetyl β-methylcholine, 0·028M; (*b*) human stroma and acetylcholine, 0·018M; (*c*) horse serum and acetylcholine, 0·018M; (*d*) horse serum and benzoylcholine, 0·006M.

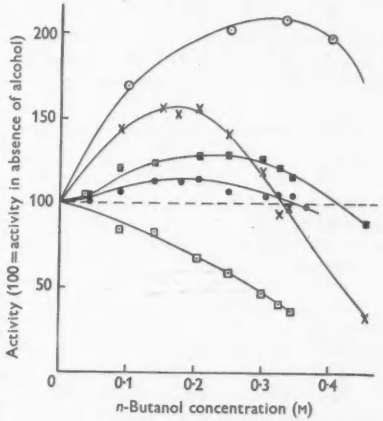

Fig. 5. Effect of varying concentrations of *n*-butanol, on the activity of cholinesterase from different sources towards different substrates. ⊙——⊙, rat-brain homogenate, 0·018M-acetylcholine; ×——×, human erythrocyte, 0·018M-acetylcholine; ■——■, horse serum, 0·006M-benzoylcholine; ●——●, rat brain, 0·028M-acetyl β-methylcholine; ⊡——⊡, horse serum, 0·018M-acetylcholine. No preliminary incubation.

The effects of varying the butanol concentration have been investigated in each case by mixing the butanol and substrate in the side arm of the Warburg flask. The enzyme activities shown in Fig. 5 have been plotted as percentages of the control activity in the absence of butanol.

With one exception, all the systems react in a qualitatively similar manner to butanol. They are all activated, the effect becoming noticeable at a butanol concentration of about 0·05M, reaching a maximum at 0·18–0·30M and changing to an inhibition at 0·35–0·45M. The maximum activation varies considerably with the nature of the enzyme and substrate, being higher where the 'true' enzyme and the physiological substrate are involved and falling off when either is replaced. The exception is the system comprising horse serum and acetylcholine. There is here no significant activation at any butanol concentration and inhibition begins at the same concentration level as activation does in the other cases and increases progressively with butanol concentration.

The marked variation with change of substrate suggests that activation is in some way competitive. The abnormal behaviour of the serum enzyme-acetylcholine system supports this, since this system is the only one of the five investigated in which inhibition of the enzyme by excess substrate does not occur (Augustinsson, 1948).

The influence of substrate concentration

The hypothesis that the activation observed might be the prevention of inhibition by excess substrate accounts for the known facts. It can also be tested in a simple manner. If the alcohol is acting as a competitive inhibitor of the inhibition by excess substrate the activation should disappear at substrate concentrations below the optimum. It is also highly probable that the alcohol will inhibit the formation of the enzyme-substrate complex which leads to hydrolysis since the molecules involved are the same; this should cause actual inhibition of the enzyme at substrate concentrations below the optimum.

The influence of substrate concentration on the effect of butanol on cholinesterase has therefore been studied in some detail. In these experiments a butanol concentration of 0·19M has been used throughout. Following Goldstein (1944) and Augustinsson (1948), the concentrations of substrate have been quoted in terms of pS (the negative logarithm of the molar substrate concentration).

The influence of acetylcholine concentration on the inhibition of the horse serum enzyme. This case is dealt with first since it involves a simpler system. According to the hypothesis put forward, no activation should be observed, since there is no inhibition by excess substrate. Competitive inhibition of the enzyme by the alcohol might, however, be expected. The experimental results indicate that inhibition occurs throughout the range of acetylcholine concentrations tested (pS, 0·74–2·74) and, furthermore, that the inhibition increases markedly as the substrate concentration is lowered. Such behaviour is typical of a competitive reversible inhibitor, and the data have therefore been subjected to Lineweaver & Burk's (1934) method of testing for competitive reversible inhibition by plotting the reciprocals of the activity and the substrate concentrations against

one another. It can be seen (Fig. 6) that the curves for the inhibited and uninhibited enzyme tend to meet at infinite substrate concentration which confirms the idea that this is basically a case of competitive inhibition.

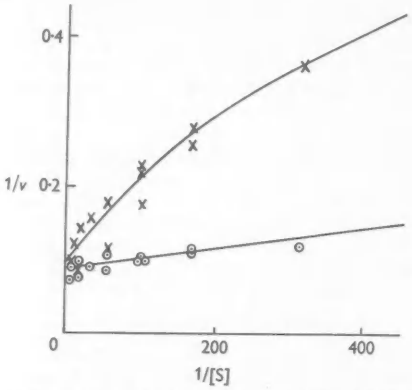

Fig. 6. Test of competitive nature of reversible inhibition in accordance with the procedure of Lineweaver & Burk (1934). Ordinates: reciprocals of activity of serum enzyme towards acetylcholine; activities in each of three experiments calculated as percentages of activity against 0·01 M-substrate. Abscissae: reciprocals of acetylcholine concentration. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of butanol.

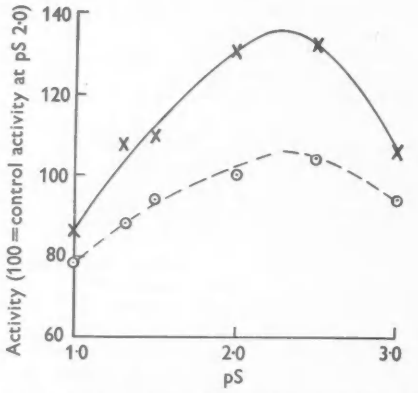

Fig. 7. Influence of benzoylcholine concentration on the effect of *n*-butanol on serum cholinesterase. *pS* = negative log of substrate concentration. ×——×, in presence of 0·19 M-*n*-butanol; ⊙——⊙, in absence of butanol.

The activation of the horse serum-benzoylcholine system. The activation already noted at pS 2·2 has now been observed over the pS range 1–3 (Fig. 7). It is maximal (30 %) at pS 2·3, i.e. about the optimum pS and falls to around 10 % at the extreme ends of the range. There is no definite shift in the optimum pS.

At pS > 2·7 a different effect is observed (Fig. 8). A rapid falling-off in enzyme activity occurs with time in contrast to the control. The significance of this will be considered later.

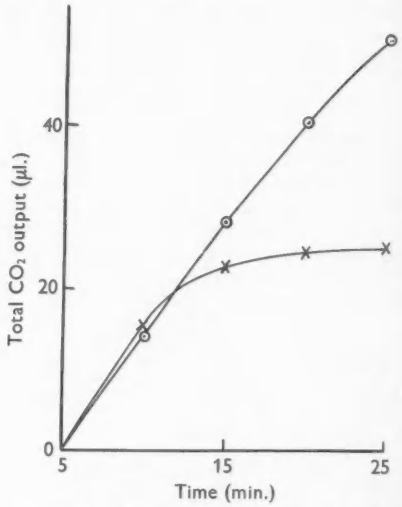

Fig. 8. The effect of *n*-butanol on the course of the hydrolysis of 0·001 M-benzoylcholine by horse serum cholinesterase. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of *n*-butanol.

The activation of the rat brain-acetylcholine system. Because the effect of butanol is most marked in the case of the true enzyme, acetylcholine being the substrate, and as this is the system of primary physiological interest, a considerable effort has been made to obtain a clear picture of the effect of substrate concentration. Unfortunately the optimum substrate concentration is very low (pS 2·6), and the experimental difficulties are therefore great.

The results of three experiments in the higher concentration range are shown in Fig. 9. The activities have been calculated in terms of the activity at pS 2·0 in the absence of butanol, in order to eliminate variations due to the differing activities of individual enzyme preparations. At high concentrations the degree of activation is almost independent of concentration (approx. 100 %), but it starts to decrease at pS 2·0.

A separate series of experiments was carried out to study the effect of butanol in the neighbourhood of the optimum substrate concentration, i.e. between pS 2 and 3. The mean results show satisfactory uniformity when calculated in terms of the control activity at pS 2·5 (Fig. 10). It seems clear that there

is a fall of 0·2–0·3 unit in the optimum pS value due to the presence of butanol, an effect which, if it were linked to an inhibition, would according to Lineweaver & Burk (1934) be indicative of a competitive

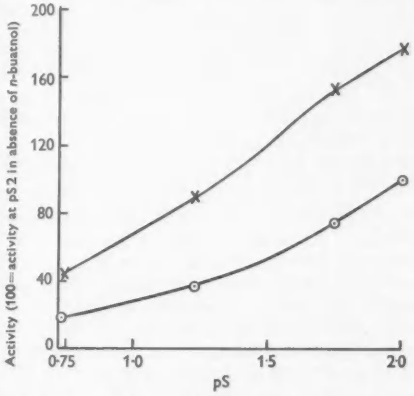

Fig. 9. Influence of acetylcholine concentration on the effect of *n*-butanol on cholinesterase (rat-brain homogenate). ×——×, in presence of 0·19 M-*n*-butanol; ⊙——⊙, in absence of *n*-butanol.

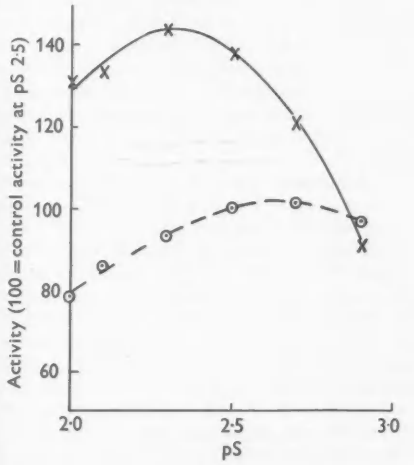

Fig. 10. The influence of acetylcholine concentration on the effect of *n*-butanol on the activity of cholinesterase (rat-brain homogenate) between *pS* 2 and 3. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of butanol.

reversible inhibition. These results also indicate that the activation decreases as the concentration falls, disappearing altogether at pS 2·9, which is in conformity with the hypothesis.

While it is not possible to measure the effect of decreasing the concentration still further, it is worth while to look closely at the results obtained at the lowest concentration (Fig. 11). The rapid decay in activity in contrast to that of the control, previously noted with the serum enzyme and benzoylcholine, can again be observed, though not to the same extent.

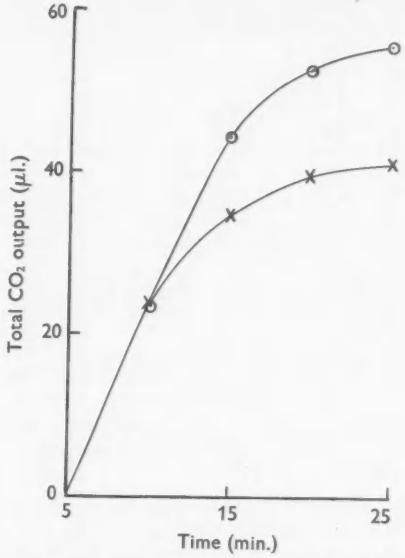

Fig. 11. The effect of *n*-butanol on the course of the hydrolysis of 0·001 M-acetylcholine by rat-brain homogenate. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of butanol.

This effect could be interpreted as an inhibition due to butanol which becomes evident only at a pS < 3, and which is observed only after the hydrolysis has proceeded for some time and so brought about the necessary lowering of the substrate concentration. This is not advanced as evidence for the hypothesis under consideration, but merely as an explanation of observed results should it be otherwise proved correct.

The activation of the rat-brain acetyl β-methylcholine system. It has become increasingly evident that, in order to test whether activation is in reality a suppression of the inhibition by excess substrate, it is necessary to work at substrate concentrations well below the optimum. In the two cases examined, the evidence has not been conclusive owing to the limitations of the experimental technique. One way out of the difficulty is to find a substrate with a higher optimum concentration. Acetyl β-methylcholine is such a substrate, since it has an optimum pS of about

2 compared with 2·4 for benzoylcholine and 2·6 for acetylcholine with the brain enzyme (Augustinsson, 1948). This substrate had been less used on account of the slight maximum activation observed (cf. Fig. 5), but, despite this, the system has been re-studied (Fig. 12).

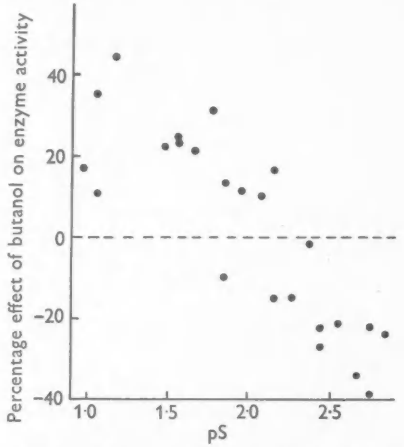

Fig. 12. Influence of concentration of acetyl β-methylcholine on the effect of *n*-butanol on cholinesterase (rat-brain homogenate).

The results show that butanol activates the enzyme between pS 1·0 and 1·8, but that at pS 2·4–2·7 there is a clear-cut inhibition. Neither the activation nor the inhibition exceeds 40 %, but the abrupt change in the sign of the phenomenon affords strong evidence for the hypothesis that the activating effect of butanol is due to depression of the inhibition by excess substrate and should change to an inhibitory effect as the substrate concentration falls below the optimum.

The effect of surface active agents

An alternative hypothesis which has been considered is that the enzyme protein is associated or aggregated in the natural state, and that alcohols by virtue of their surface-active properties cause dissociation, with consequent exposure of additional active centres.

If this were the case compounds with highly specific surface-active properties such as the anionic and kationic soaps should bring about activation at remarkably low concentrations. It has been found, however, that dodecylpyridinium bromide and sodium dodecylsulphonate show no signs of activating cholinesterases. They do inhibit the enzymes at concentrations of the order of 10^{-4} M (Table 2), the kationic soap being the stronger inhibitor of the two by a factor of about 10.

Table 2. *The effect of surface active agents on cholinesterases*

Enzyme ...	Rat brain	Horse serum	
Substrate ...	0·018M-acetylcholine	0·018M-acetylcholine	0·006M-benzoylcholine
Surface active agent	50% inhibition concn. (no pre-incubation)		
Dodecylpyridinium bromide	$4{\cdot}3 \times 10^{-4}$	$4{\cdot}4 \times 10^{-5}$	$3{\cdot}5 \times 10^{-4}$
Sodium dodecylsulphonate	$1{\cdot}7 \times 10^{-3}$	$8{\cdot}2 \times 10^{-4}$	$1{\cdot}6 \times 10^{-3}$

The absence of activation, though not necessarily conclusive, is evidence against the dissociation hypothesis. The behaviour of the serum enzyme towards the two substrates affords additional strong evidence against this hypothesis. A theory based on simple dissociation could hardly account for the fact that the hydrolysis of acetylcholine is inhibited while that of benzoylcholine is activated.

DISCUSSION

The picture which seems to emerge from the foregoing is that the activation of cholinesterase by alcohols is not a positive but a negative effect, in which the alcohols act as inhibitors of the enzyme at all times, but can, in certain circumstances, interfere with another inhibitory process to such an extent as to cause an apparent activation. This type of activation appears to be quite distinct from the two types of activation previously described (Baldwin, 1947).

Objections may be raised on the grounds that the enzyme preparations used were not purified and may have contained both types of cholinesterase; it might then be suggested that differential inhibition coupled with the different optimum substrate concentrations might give the appearance of activation by alcohols.

Mendel, Mundell & Rudney (1943) have, however, shown that horse serum hydrolyses acetyl β-methylcholine at one-tenth of the rate at which it hydrolyses benzoylcholine. From Table 2 of their paper it can be calculated that this is equivalent to an activity of the 'true' enzyme in terms of acetylcholine splitting activity of 2 % of that of the 'pseudo' enzyme. The human erythrocyte enzyme used in this work was purified in such a manner as to free the stroma from other components. Like Mendel & Rudney's (1943) preparation it should be free from serum esterase. Ord & Thompson (1950) have put forward evidence, based on the activity against the specific substrates, that rat brain contains less than 10 % of the 'pseudo' cholinesterase.

Since, in the experiments reported, the specific substrates have been used with enzyme preparations

which are known to act specifically on them, the criticism that the activity of a mixture of enzymes is being measured does not appear to us to be valid. The question of aliesterase activity (Adams & Whittaker, 1949; Richter & Croft, 1942) does not appear to be involved.

The inhibition of cholinesterase by alcohols observed by the majority of the earlier workers is to be expected since, without exception, they employed biological assay techniques to measure the rate of destruction of acetylcholine; these rendered desirable the use of very low initial acetylcholine concentrations. Those employed were: Plattner & Galehr (1928), $1{\cdot}4 \times 10^{-4}$M; Bernheim & Bernheim (1936), $3{\cdot}7 \times 10^{-4}$M; Heim & Fahr (1940), $1{\cdot}9$–$5{\cdot}7 \times 10^{-5}$M; Genuit & Labenz (1941), $3{\cdot}7$–$9{\cdot}3 \times 10^{-7}$M.

It is difficult to understand the activation observed by Heim & Fahr, since the acetylcholine concentration they employed is well below the optimum.

It would probably be correct to say that the activation of cholinesterase by alcohols is of no physiological significance since, although there is no convincing evidence available regarding the actual acetylcholine concentration which develops at the cholinergic nerve endings, it must be relatively low judged by the total acetylcholine liberated.

Our results are perhaps of more significance from the point of view of enzyme technique, particularly in connexion with the study of inhibition. Augustinsson (1949) has recently stressed the point that the degree of inhibition brought about by a reversible competitive inhibitor is markedly dependent on the substrate concentration employed, though he did not go on to suggest that a negative inhibition could be obtained by a mere variation of the substrate concentration.

Our findings confirm his view that the choice of a single substrate concentration for the measurement of enzyme activity is extremely difficult in the case of those enzymes which are inhibited by excess substrate. It is not suggested that all the inhibitors of enzyme activity will also interfere with the inhibition by excess substrate, but, since it appears to have occurred in one set of cases, it is not permissible to assume that it does not occur in others. Richter & Croft (1942) have, for instance, reported the activation of cholinesterases by quinine and atoxyl. It does not seem impossible that interference could occur to an extent that would not show up obviously as an activation.

On these grounds, it is suggested that, in studies on the reversible inhibition of those enzymes which are also inhibited by excess substrate, a substrate concentration or concentrations fairly well below the optimum should be chosen. This would be unnecessary, however, if it could be proved beforehand that the inhibitor did not interfere with the inhibition by excess substrate.

SUMMARY

1. The effect of alcohols on cholinesterases has been investigated. They have been found to bring about activation of the enzyme up to an optimum concentration which decreases as the chain length of the alcohol increases. At higher concentrations inactivation occurs.

2. The activation is practically instantaneous and reversible, the inactivation slower and irreversible.

3. No activation of the hydrolysis of the acetylcholine by the serum enzyme is observed, only a competitive reversible inhibition over the same alcohol concentration range.

4. The hypothesis that the activation is primarily an interference with the inhibition by excess substrate, has been examined by studying the effect of substrate concentration. It has been shown that, in the hydrolysis of acetyl β-methylcholine by the brain enzyme, the activation observed at high substrate concentrations changes at low substrate concentrations to an inhibition.

5. Specific surface-active agents do not cause activation of the enzyme.

6. The importance of the substrate concentration in the study of cholinesterase activity is underlined by this work.

We are indebted to the Chief Scientist, Ministry of Supply, for permission to publish this paper, and to Mr D. R. Davies for constant encouragement and advice. We wish to express our thanks to Messrs Imperial Chemical Industries, Ltd., Blackley, for samples of surface-active agents. Thanks are also due to Miss L. A. Barker and Mr T. C. Askey for technical assistance in certain experiments.

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Composition of Ox Lens Capsule

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The behaviour and constitution of the ox lens capsule has been investigated to see whether it is made up of a single protein containing carbohydrate or of protein mixed with polysaccharide.

The lens capsule completely encloses the lens and is a non-cellular laminar membrane thought to be secreted by the lens epithelium. It is elastic and when cut rolls back on itself as if the outer layers were under tension. Mammalian lens capsules can easily be stripped off like a cellophan wrapper and are tough and withstand handling. The adult lens has a single layer of epithelial cells on the front face only and it is here that the capsule is thickest. The posterior capsule is thinner, being laid down in embryonic life when the lens epithelium extends to the back surface. The evidence that the capsule is secreted by the epithelium comes from the work of Shirmer (1889), who showed that injury to the capsule and epithelium of the lens in the adult rabbit was followed by repair and a thickening of the capsule over the site of injury where the epithelial cells proliferate. This was confirmed by Bakker (1936–7) who, after injuring the rabbit lens *in situ*, removed it and watched the repair of the capsule in a tissue culture of the whole lens.

Mörner (1894) showed that the ox lens capsule contained about 10% of carbohydrate. Krause (1933) found N, 14%, S, 0·84%, and considered that the capsule was a distinct insoluble protein unlike the other widely distributed insoluble proteins of the body. Hertel (1933) took X-ray diffraction photographs of the capsule and found the picture was like that of unoriented collagen. Washed lens capsule was used in all these analyses and was considered to be a single protein. If this assumption is correct the lens capsule protein is unique in giving the X-ray diffraction pattern of collagen and yet containing this large amount of carbohydrate. It is also peculiar if it is a type of collagen and yet is produced by epithelial cells.

METHODS

Preparation of lens capsule. Ox eyes were dissected within 1 hr. of death. The lens was removed in its capsule and this was then stripped off. The capsules were suspended in water and stirred for several days with many changes of water, in order to remove attached epithelial cells and lens fibres. Such treatment did not alter the appearance of the capsules, but would presumably remove any soluble substances present. The capsules were then either acetone-dried or used fresh.

Total nitrogen. This was estimated by micro-Kjeldahl followed by distillation and titration, as described by Markham (1942).

Hexosamine. Elson & Morgan (1933).

Total carbohydrate. The orcin colorimetric method described by N. W. Pirie (1936) was used.

Reducing sugars. Hagedorn & Jensen (1923).

Partition chromatography of amino-acids. The upward method described by Williams & Kirby (1948) was used.

Partition chromatography of sugars. The upward method was used with the developers and spraying solutions described by Jermyn & Isherwood (1949) and Partridge (1949).

RESULTS

Ox lens capsule, prepared as described, contained 14% N, 9–10% carbohydrate, estimated colorimetrically, or 9% reducing sugars after hydrolysis. It also contained about 1% hexosamine. This small amount is difficult to estimate owing to charring during hydrolysis. No phosphorus was found.

General properties. The properties of the capsule were studied to see how far they tallied with those of collagen. Lens capsule is insoluble in boiling water, but goes into solution readily at about pH 3. Such solutions, after dialysis, set to weak jellies at a concentration of about 1%. The capsule does not gel as readily as gelatin derived from skin or tendon

collagen, but the fact that it will form a gel after slight hydrolysis is in favour of it containing a protein like collagen. It also resembles collagen (Theis, 1946) in having the same definite marked shrinkage temperature between 60 and 65°.

Lens capsule is also soluble in 0·1 N-sodium hydroxide or 40 % urea at 100°. Formamide, lactic acid, 40 % urea, 10 % calcium chloride or 90–95 % (w/v) phenol do not dissolve it at 37°. Formic acid dissolves the capsule at 37°. Considerable swelling of the capsule occurred in phenol, lactic acid and urea solutions at 37°, although no solution took place. Within this range the solubility and swelling of the capsule is the same as that of collagen. Many types of collagen are insoluble in boiling water, but dissolve in weak acid.

X-ray diffraction photograph. The X-ray diagram of dried stretched lens capsule was studied by Dr D. M. Crowfoot, and the results of Hertel (1933) were confirmed and extended. The picture conformed in general to that given by partly oriented collagen. The characteristic meridional spacing of 2·85 A. was present, together with the equatorial spacing of 4·3 A., but the longer spacings differed from those of typical collagen. This leaves open the possibilities that the lens capsule contains another protein besides typical collagen, or that it consists of an atypical collagen. The chemical analysis is more in favour of the second possibility. Attempts to decide the question were made by examining the amino-acid composition of the lens capsule and by trying to separate the carbohydrate by extraction and digestion methods.

Composition of lens capsule. A comparison of the constituent amino-acids with those of collagen was made. Sections of the eye stained with Millon's reagent showed that the lens capsule stained an intense red, far stronger than other parts of the eye. Estimation of tyrosine in lens capsule and cornea collagen by the method described by Lugg (1938) showed that the lens capsule contained 2·1 % and cornea collagen 1·0 % tyrosine, calculated on a moisture- and ash-free basis. Tristram (1946) found 1·4 % tyrosine in skin collagen.

A general survey of amino-acid composition was then made using paper partition chromatography. Lens capsule, cornea collagen, gelatin and sometimes casein were hydrolysed with 6 N-hydrochloric acid overnight at 110° and the acid removed *in vacuo* in the usual way. Upward chromatography, as described by Williams & Kirby (1948), was used. Water-saturated phenol, sometimes with additions of 3 % ammonia or cupron were used as developers. The dried papers were sprayed with ninhydrin.

The general pattern of the lens capsule chromatogram was the same as that of cornea collagen and gelatin and quite unlike that of casein. The glycine spot was very prominent and proline and hydroxyproline spots were present. There seemed a larger spot corresponding to the leucines and phenylalanine in the lens capsule chromatogram. The difference in tyrosine content did not show up. All that one can say from this result is that the capsule showed some similarities to, and no marked difference from, collagen and gelatin.

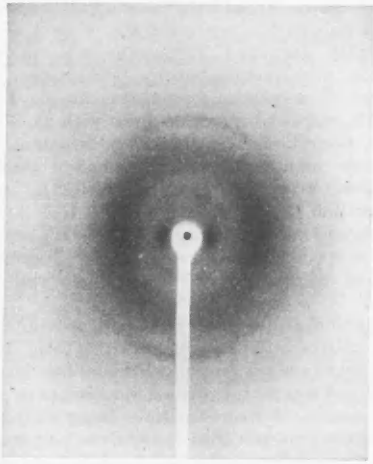

Fig. 1. X-ray photograph of dried ox lens capsule. Stretched preparation showing some orientation of fibres. Photograph taken with cylindrical camera, radius 3·0 cm.

In order to try and separate the carbohydrate, capsules were collected and extracted with 10 % (w/v) sodium chloride followed by 0·1 M-dipotassium hydrogen phosphate, with phenol 90–95 % (w/v), 40 % urea, or 10 % calcium chloride at 37°, extractives that have been used to separate polysaccharides from protein in other cases. Sodium chloride, followed by alkaline phosphate extraction brought out very little, the other extractives removed 10–20 % by weight of the capsule. The carbohydrate content of the extracted material was, however, usually rather less (5–7 %) than that of the original capsule. The undissolved residue had 10–11 % carbohydrate. Removal of carbohydrate was therefore not achieved by these methods. Mörner (1894) found that 0·1 % potassium hydroxide failed to extract the carbohydrate from lens capsule.

Extraction of carbohydrate from intact capsule having failed, the effect of enzyme digestion followed by fractionation was next tried. Pepsin, trypsin and collagenase dissolve lens capsule, leaving only a trace of insoluble material. Native skin collagen is dissolved by pepsin and collagenase, but is only very slightly digested and not then taken into solution by trypsin. Bidwell & van Heyningen (1948), who

described the properties of collagenase, found that it hydrolysed only collagen, gelatin and reticulin of all proteins investigated. It is, therefore, interesting that this enzyme digests the lens capsule, and collagenase digestion was used in an attempt to separate carbohydrate from protein.

Lens capsule (200 mg.) was digested with collagenase at 37° for several weeks and the digest was then dialysed. About half was dialysable and contained only 5–6% carbohydrate while the non-dialysable part had 13%. The non-dialysable fraction was fractionated by precipitation with ammonium sulphate. Precipitates having 10, 12 and 9% carbohydrate came down with 25, 50 and 100% saturation. The dialysed supernatant from complete saturation with ammonium sulphate contained 5·3 mg. material having 22% carbohydrate and 4% hexosamine.

Collagenase digestion does not, therefore, separate protein from carbohydrate, which appears distributed unevenly among the protein split products.

Digestion with either crystalline trypsin (Armour Chemicals) or with pancreatin (British Drug Houses Ltd.) led to the same result. Ethanol fractionation of a digest was no better than ammonium sulphate precipitation, the carbohydrate being distributed among the products of hydrolysis, the best fraction being that precipitated by 10 vol. ethanol and having 19·4% carbohydrate and 7·9% N. No hydrolytic method was successful in separating carbohydrate from protein, so the next step seemed to be to find out what type of sugar was present in the carbohydrate.

Identification of the carbohydrate. The constituent sugars of the lens capsule carbohydrate were identified by paper partition chromatography using the upward method. Lens capsule (150 mg.) was hydrolysed by heating for 3 hr. in a boiling-water bath with 0·5N-hydrochloric acid. This time of hydrolysis gave a maximum value of 9–11% reducing sugar. The acid solution was dialysed and the dialysate dried and redissolved in a small volume of water and brought to pH 6. This was applied to a column containing 0·5g. washed Zeo-Karb 215. The dried effluent (95 mg.) was dissolved, brought to pH 4 and again passed through a column. The dried effluent (72 mg.) had 14% total carbohydrate. It was extracted with ethanol at room temperature and the extract dried. This material (37 mg.) had a total carbohydrate content of 18% while the material not soluble in ethanol had 11% carbohydrate. The material soluble in ethanol was analysed chromatographically using the ethyl acetate-pyridine-water mixture (2:1:2 or 5:2:5) described by Jermyn & Isherwood (1949) as developer, and spraying the dried papers with aniline hydrogen phthalate (Partridge, 1949) to show up the spots. Repeated upward runs were done in order to get good separation of spots, so that R_F values could not be measured. Marker spots of different sugar solutions were always used.

The lens capsule fraction gave two not completely separated spots corresponding in position to glucose and galactose. The glucose spot was much the larger of the two. This result was confirmed using a lens capsule fraction got by direct ethanol extraction of the dried acid hydrolysate followed by Zeo-Karb treatment of an aqueous solution of this material. No trace of any other sugar appeared on the chromatogram, but the final yield of carbohydrate usually only accounted for between 40 and 50% of the carbohydrate of the lens capsule. Zeo-Karb treatment removed considerable carbohydrate.

Colorimetric estimations agreed in showing the presence of a small amount of galactose. N. W. Pirie (1936) showed that galactose gave a red colour with orcin at 55° within 3 min. of heating, whereas other sugars gave yellower colours and only at higher temperatures. If lens capsule was heated with orcin at 55° a definite pink colour appeared in 3 min. and was much intensified and became orange when the solution was heated to boiling point. The colour given by the lens capsule with the carbazole reagent of Gurin & Hood (1939) also corresponded to a mixture of glucose and galactose.

DISCUSSION

No separation of constituents from the lens capsule has been achieved, and the conclusion still remains valid that it is probably composed of a single major constituent, an insoluble protein which contains about 10% of carbohydrate. It has many characteristics of collagen, but the two are not identical. From the point of view of the physiology of the eye the difference between the lens capsule protein and the closely associated fibrillar protein of the vitreous body is interesting. The protein of the vitreous body can be entirely separated from carbohydrate, and Pirie, Schmidt & Waters (1948) identified it with normal collagen from a study of its physical properties and chemical analysis. The cells of origin of the fibrillar protein of the vitreous body are not known.

The lens capsule is deeply stained by the Hotchkiss (1948) method for the detection of polysaccharide in tissue sections. Other structureless membranes such as Descemet's membrane of the cornea, Bruch's membrane of the retina also stain deeply. A basement membrane lining the whole vascular tree can also be demonstrated by this method (Day, 1950). It is therefore possible that insoluble proteins rich in carbohydrate are present in many membranes of the body, and that the lens capsule is not a unique structure. Such proteins would be difficult to detect

if they occurred in small amounts together with collagen. It is noticeable that the mixture of sugars in the lens capsule carbohydrate, glucose and galactose, are those which have been found in the 0·65 % of carbohydrate which it is difficult to remove from skin collagen (Schneider, 1937). The capsule also probably contains a small amount of hexosamine. Hydrolysates of whole capsule give a value of about 1 % and this might not be thought significant, but fractions prepared by enzyme digestion have been found to contain 2–4 % hexosamine, which makes it likely that a small amount is present in the capsule itself.

SUMMARY

1. Further analysis of ox lens capsule has failed to separate it into separate constituents.

2. It appears to be composed of a protein similar to collagen in many of its physical and chemical properties, but containing about 10 % of carbohydrate.

3. The carbohydrate consists mainly of glucose with a small amount of galactose.

I am greatly indebted to Dr D. M. Crowfoot, F.R.S., for the X-ray photograph of lens capsule and for her interpretation of it for me. I have pleasure in thanking Dr E. Bidwell for the preparation of collagenase.

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The Use of Radioactive Isotopes in Immunological Investigations

6. SOME OBSERVATIONS ON PRECIPITIN INHIBITION TESTS AND ON ANTIBODY VALENCE

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Landsteiner (1920) showed that the precipitation of azoproteins by their antisera could be partially or completely inhibited by simple substances structurally identical with, or very similar to, the compound which had been linked to the protein. This type of competitive inhibition has been used extensively by Landsteiner and his colleagues, and by many other immunologists, for the study of the serological specificity of conjugated proteins and other antigens (see the reviews by Marrack, 1938; Landsteiner, 1945; Boyd, 1947). It has also been shown that the immunological specificity of iodinated proteins is due to their 3:5-diiodotyrosine groups (Wormall, 1930), since 3:5-diiodotyrosine (and to a lesser degree the corresponding bromo-compound) specifically inhibits the reaction between iodinated proteins and their antibodies; further evidence of this specific inhibitory action of 3:5-diiodotyrosine, and compounds of similar constitution and structure, was obtained by Jacobs (1932), and Snapper & Grunbaum (1936). Landsteiner suggested that the inhibitor specifically combined with the serologically active groups of the appropriate antibodies, thus preventing union of antibody and antigen to give precipitation. Proof of this union of specific inhibitor and antibodies was obtained by Marrack & Smith (1932) and Haurowitz & Breinl (1933). Further support was given by the observation that relatively simple azo dyes may (*a*) give precipitates with antisera to the corresponding dye-azoprotein,

and (*b*) cause anaphylactic shock when injected into guinea pigs which had been sensitized with the corresponding azoprotein.

Although serological inhibition tests have been extensively used for the qualitative study of precipitin and complement-fixation tests, few quantitative investigations have been made in this field (cf. however, Woolf, 1941). We have, therefore, studied the composition of precipitates separating from mixtures of iodinated proteins and their antibodies in the presence of varying amounts of specific inhibitor (3:5-diiodotyrosine). The results are discussed in relation to antibody valence.

The valence of antibodies, i.e. the number of specific combining groups or binding sites in the antibody molecule, has been widely studied, and two different views are held (for references see Boyd, 1947; Landsteiner, 1945; Grabar, 1950). Haurowitz, Boyd and others believe, that antibodies are univalent whilst Pauling and many other workers maintain that antibodies are bi- or multivalent. The 'lattice theory' of the precipitin reaction (Marrack, 1938) requires that the antibody molecule should have at least two groups capable of combining with antigen, and Pauling and his colleagues have obtained evidence that most of the molecules of antibody are at least bivalent (cf. for example, Pauling, 1940; Pauling, Pressman & Ikeda, 1942; Pauling, Pressman & Campbell, 1944).

Antigen molecules are usually multivalent, and our iodinated protein antigens may contain up to sixty-five combining or determinant groups per molecule. Thus it seems probable, for reasons given later in this paper, that if a 'lattice structure' containing bi- or multi-valent antibody is precipitated in the presence of concentrations of specific inhibitor sufficient to cause only partial inhibition of precipitation, there should be a significant amount of the inhibitor in the precipitate. Investigations designed to test this possibility were therefore undertaken, and are described below. Brief accounts of some of the results have been given elsewhere (Banks, Francis, Mulligan & Wormall, 1949, 1950*a*).

EXPERIMENTAL

The non-radioactive iodinated serum globulins used for immunization and the ^{131}I-containing serum globulins used as test antigens were prepared as described (Banks, Francis, Mulligan & Wormall, 1950*b*). The preparation of other antigens is described below.

Iodinated rabbit serum proteins. 1% (w/v) KI solution (5·5 ml.) was treated with 22 μc. of ^{131}I as NaI and I_2 was liberated by adding 0·1 N-KIO_3 (4 ml.) and then acidifying the mixture with HCl. The liberated I_2 was extracted with ether and the ethereal solution was added slowly to 2 ml. rabbit serum diluted with 4 ml. 2·5 N-NH_3. The ether was then rapidly removed *in vacuo* and the iodinated proteins were precipitated by adding acetic acid. The proteins were again dissolved at pH 7·5 and precipitated by addition of acetic acid. This was repeated once more. The final precipitate was dissolved in 0·9% NaCl with the addition of 2 N-Na_2CO_3 to bring to pH 7·5. The non-radioactive iodinated rabbit serum proteins were prepared similarly; both preparations contained about 14% I.

3:5-*Diiodotyrosine containing* ^{131}I. This was prepared by the method of Harington (1928) with slight modifications introduced to reduce the loss of ^{131}I. A solution of 10% (w/v) KI (7·7 ml.) was added to 528 μc. of ^{131}I (as NaI), followed by N-KIO_3 (5·5 ml.). A slight excess of N-HCl was then added, and the liberated I_2 was extracted with ether. The ethereal solution was then gradually added, with shaking, to a solution of tyrosine (0·25 g.) in NH_3 (sp.gr. 0·880) (5·5 ml.). When all the I_2 had been added the aqueous solution was separated and kept in a desiccator over conc. H_2SO_4 until practically all the NH_3 had been removed. The resulting brown sludge was dissolved in water (15 ml.) containing a few drops of N-NaOH, and the solution decolorized with charcoal. N-Acetic acid was then added to precipitate the 3:5-diiodotyrosine, which was then washed with ice-cold water and precipitated again, in crystalline form, by acidifying a slightly alkaline solution of the compound. The yield was 0·432 g. (72% of theory). With a thin-window bell-type counter, it gave 100 counts/μg./min. For the inhibition tests a stock 0·02 M-solution, in 0·9% NaCl at pH 7·5, was prepared.

Antisera. These were obtained by the intramuscular injection of alum-precipitated antigens into rabbits (Proom, 1943). Fresh unfiltered antisera were used in the experiments recorded in Tables 1 and 3, and 'Berkefeld-filtered' and stored serum for the experiments recorded in Table 2 and Fig. 1 (cf. Banks *et al.* 1950*b*).

Precipitin tests. These were carried out as described by Banks *et al.* (1950*b*).

Precipitin-inhibition tests. These followed the general method of Landsteiner (1920), as used by Wormall (1930) for the inhibition of the iodinated protein antigen-antibody reaction by 3:5-diiodotyrosine. The inhibitor was added to the antiserum and the antigen solution was then added to the mixture. Where D_2O was used, it was added before the antigen. Preliminary tests were made with non-radioactive 3:5-diiodotyrosine to determine the amount of inhibitor required to give suitable partial inhibition of precipitate formation. Full details of these experiments are given with the tables or in the appropriate parts of the text.

Radioactivity determinations. These were made as described by Banks *et al.* (1950*b*), using side-window or bell-type thin end-window Geiger-Müller counters.

Molecular ratios. In calculating the molecular ratios, the value 165,000 was used for the molecular weight and 16·0% for the N content of antibodies and the mixed serum globulins. It is recognized that antibody molecules are not necessarily homogeneous, but in these investigations we were concerned with the average precipitating antibody molecules. The molecular weight of 3:5-diiodotyrosine is 433.

D_2O *determinations.* The heavy water samples were separated from the protein solutions by vacuum sublimation and condensation at about $-70°$, and their purity was checked by measurement of refractive index. The D_2O concentrations were then determined by the falling drop method (Barbour & Hamilton, 1926) using the apparatus described by Popják (1950).

Total iodine determinations. These were made by the method of Shahrokh (1943). Under the conditions of our experiments we have found this satisfactory for the determination of 10–100 μg. of iodine, with an accuracy of ± 1 μg.

RESULTS

Iodine content of precipitates separating from iodinated protein antigen-antibody mixtures containing 3:5-*diiodotyrosine*

In one typical experiment the precipitation of iodinated proteins by an antiserum to iodinated horse serum globulin was partially inhibited by 3:5-diiodotyrosine and the precipitates were analysed for antigen and inhibitor. In one set of mixtures the antigen was labelled with ^{131}I and in a parallel set the inhibitor only was radioactive. To exclude as far as possible interference due to species differences, the test antigen was prepared from rabbit serum proteins; the amount of antigen used was that required to give approximately maximal precipitation of antibody in the absence of inhibitor.

Samples (2 ml.) of the antiserum were treated with 3:5-diiodotyrosine (1 or 2 ml. of a 0·0025 or 0·01 M-solution in 0·9% NaCl), and 0·9% NaCl to make a total volume of 4 ml. The antigen solution (4 ml. of a 0·043% solution of ^{131}I-containing iodinated rabbit serum proteins or a 0·048% solution of a similarly prepared non-radioactive antigen) was added to each tube. The mixtures were stirred well and kept at 37° for 3 hr., followed by 15–18 hr. at about 4°. They were then centrifuged and the sides of each tube were washed with six drops of 0·9% NaCl, care being taken to avoid disturbing the precipitate. The precipitates were then dissolved in a few drops of approximately 0·5 N-NaOH, and ^{131}I and total I determinations made as described above. Control mixtures of normal rabbit serum plus radioactive antigen and/or inhibitor gave no precipitate.

In these experiments the amount of antigen precipitated in the mixtures containing radioactive antigen and non-radioactive inhibitor was 36–54% of that precipitated in the absence of inhibitor, and the corresponding range for the non-radioactive antigen series was 38–60% (Table 1). In the former series there was good agreement between the iodine contents of the precipitates as determined by ^{131}I and by total iodine. This suggests that all the iodine in the precipitates was due to antigen. More definite evidence of this was obtained from the analyses of precipitates from the mixtures containing radioactive diiodotyrosine and non-radioactive antigen, for these precipitates contained no detectable ^{131}I. The molecular weights of the antigen proteins are, however, about 150–400 times that of diiodotyrosine; these results with radio-diiodotyrosine of relatively low specific activity, and with precipitates which were not completely freed from supernatant solution, would not, therefore, exclude the possibility of the precipitate containing a few diiodotyrosine molecules per molecule of antigen or antibody.

In the experiment recorded in Table 1, the antibody:antigen ratio was not determined, but other experiments made with the same antiserum, but without inhibitor, gave ratios ranging from 4·1 (antigen excess) to 12·6 (antibody excess) for precipitates obtained with iodinated rabbit serum proteins as test antigen (assuming a mean molecular weight of 10^5 for the serum proteins), and a corresponding range of 1·5–5·0 with iodinated ovalbumin.

Table 1. *Iodine content of iodinated protein antigen-antibody precipitates from systems containing* 3:5-*diiodotyrosine*

Antigen: Iodinated rabbit serum proteins.
Antiserum: Rabbit antiserum to iodinated horse serum globulin.
Inhibitor: 3:5-Diiodotyrosine (DIT).

(Weights given are for precipitates from 2 ml. of antiserum. Other experimental details are given in the text.)

Component labelled with ^{131}I	DIT added (μg.)	Degree* of precipitation	Composition of precipitate: Iodine content: By ^{131}I (μg.)	Composition of precipitate: Iodine content: Total I (μg.)	Amount of antigen as percentage of that added
Antigen	0	+ + ±	26·6	29·2	11·3
	1082	+ ±	15·2	16·0	6·4
	2165	+	13·9	14·2	5·9
	4330	±	12·1	12·4	5·1
	8660	tr.	10·6	10·2	4·5
DIT	0	+ + ±	0	30·9	11·6
	1082	+ ±	0†	18·3	6·9
	2165	+	0†	16·9	6·3
	4330	±	0†	13·8	5·2
	8660	tr.	0†	11·8	4·4

* Taken from a parallel set of precipitin tests made with one-twentieth the volumes of solutions used in the quantitative experiment. The readings given are those after 3 hr. at 37°, and the results are recorded as follows (in increasing degrees of precipitation): tr. (trace), ±, +, + ±, + +, + + ±.

† Counts within variation of background and therefore not quantitative, but indicate certainly less than 3 μg. iodine.

Composition of iodinated protein antigen-antibody precipitates from systems containing 3:5-diiodotyrosine

In further inhibition experiments the washed specific precipitates were analysed for antigen and antibody.

Samples (0·5–3·0 ml.) of a rabbit antiserum to iodinated human serum globulin were treated with 0·01 M-3:5-diiodotyrosine (0·05, 0·083 or 0·133 ml./ml. antiserum) and then mixed with a solution of iodinated horse serum globulin containing ^{131}I (0·01–0·4 ml. of a solution containing 1·037 mg. N/ml./ml. antiserum). Sufficient 0·9% NaCl was added to make the total volume twice the original serum volume. The mixtures were kept at 37° for 1 hr. and then at 0–4° overnight; they were then washed twice with 0·25 ml. portions of 0·9% NaCl, dissolved in N-NaOH (0·5 ml.) and made up to 2·5 ml. Samples of these solutions (1–2 and 0·3 ml. respectively) were used for total N and radioactivity determinations.

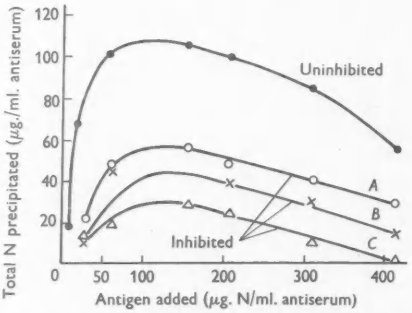

Fig. 1. Total N in precipitates from the iodinated protein antigen-antibody system in the presence of 3:5-diiodotyrosine (inhibitor). Antigen, iodinated horse serum globulin. Antiserum, rabbit antiserum to iodinated human serum globulin. *A*, least inhibitor; *C*, most inhibitor. Further experimental details are given in Table 2.

The results show that, in some respects, the effect of adding the specific inhibitor is equivalent to reducing the amount of antibody available for combination with antigen. This causes not only a decrease in the amount of precipitate obtained (Fig. 1), but also, with most of the antigen-antibody mixtures examined, a decrease in the antibody: antigen ratio in the precipitate (Table 2). As increasing amounts of antigen are used, however, and the ratio tends towards its lower limit, this latter effect becomes less marked, and the inhibited and uninhibited systems ultimately give the same ratio.

It was not practicable to investigate fully the precipitation in inhibited systems containing very small amounts of antigen, since the amount of precipitate obtained was too small for complete analysis: for example, addition of 31 μg. antigen produced, per ml. antiserum in an inhibited system, precipitates containing only 2·5 and 6·5 μg. antigen and antibody nitrogen respectively, compared with a precipitate containing about 84 μg. nitrogen in the uninhibited system.

Table 2. *Composition of washed precipitates separating from iodinated protein antigen-antibody mixtures in the presence of 3:5-diiodotyrosine*

Antigen: Iodinated horse serum globulin (containing ^{131}I).
Antiserum: Rabbit antiserum to iodinated human serum globulin.
Inhibitor: 3:5-diiodotyrosine (DIT).

(The values in brackets are probably unreliable.)

Antigen added (μg. N/ml. serum)	Antibody N : antigen N ratios of the precipitates			
	Uninhibited (no DIT)	Partially inhibited		
		(*A*)*	(*B*)*	(*C*)*
10·4	8·1	—	—	—
20·8	5·9	—	—	—
31·1	—	2·7	2·6	(4·1)
62·3	2·9	1·9	1·9	2·3
156	1·7	1·3	—	1·4
208	1·4	1·0	1·1	1·4
311	1·1	(0·8)	1·1	1·2
415	1·3	1·1	1·2	—

* The concentrations of 3:5-diiodotyrosine in series (*A*), (*B*) and (*C*) were 2·5, 4·16 and $6·7 \times 10^{-4}$ M, respectively.

The use of an additional tracer (heavy water) to measure the amount of supernatant solution adhering to serological precipitates

In many of our investigations we have sought to determine whether or not combined specific inhibitor is present in serological precipitates which separate from mixtures of antigen and antibody with sufficient inhibitor to effect only partial inhibition. The concentration of inhibitor needed for measurable inhibition is relatively large, and there is, therefore, a considerable amount of inhibitor in the supernatant solution which adheres to the precipitate. This makes it difficult for the amount of combined inhibitor to be determined. Serological precipitates can often be washed with cold 0·9% sodium chloride solution without serious loss of antigen or antibody, but it should not be assumed that this will also apply to any combined inhibitor in the complex. We decided, therefore, not to wash the precipitates in these particular inhibition tests, and we sought some method which would enable us to estimate the amount of adhering, as distinct from combined, inhibitor, in the unwashed precipitates. It was essential that any method adopted for this purpose should not interfere with the determination of antibody, antigen and total inhibitor in the precipitate, i.e. with total nitrogen, and, in the experiments described here, total iodine and ^{131}I determinations.

We found that our object could be achieved by using heavy water as an additional tracer. Deuterium oxide was added to the antigen-antibody-inhibitor mixture in amounts sufficient to render possible a determination of the deuterium oxide content of a measured fraction of the specific precipitate. From the results we calculated the amount of supernatant solution, and hence the uncombined inhibitor, associated with the precipitate; from this value and the nitrogen, iodine and ^{131}I contents, the amounts of antibody, antigen and combined inhibitor in the precipitate could be calculated. Preliminary experiments showed that, under the conditions of our experiments, a final concentration of 5% deuterium oxide in the mixture of antigen, antibody and inhibitor was suitable for this 'additional tracer' technique. A typical experiment was carried out as follows.

Samples (6 ml.) of a rabbit antiserum to iodinated horse serum globulin were mixed with NaCl solution containing heavy water (1 ml. containing 0·9% NaCl and 50% (w/v) D_2O) and ^{131}I-containing 3:5-diiodotyrosine solution (0·25, 0·75 or 2·0 ml. of 0·01 M). The antigen (1 ml. of 0·6% iodinated human serum globulin) and sufficient 0·9% NaCl to give a total volume of 10 ml. were then added. The mixtures were kept at 37° for 3 hr. and then at 0–4° overnight; the precipitates were separated by centrifuging, and the sides of the tubes were cleaned with filter paper, care being taken to avoid disturbing the precipitate. These unwashed precipitates were then dissolved in water containing a few drops of 0·5 N-NaOH and the volume made up to 3 ml. Total N and ^{131}I determinations were made on 0·1 and 1·0 ml. samples respectively, and the D_2O contents of these solutions, and of the supernatant solutions separated from the precipitates, were also determined.

The results showed that the amount of combined inhibitor (3:5-diiodotyrosine) in the antigen-antibody complex was very small and probably zero (Table 3). The calculated molecular ratio of combined inhibitor:antibody was sometimes zero, and in no case was it greater than 1:20.

DISCUSSION

For the quantitative study of serological inhibition tests radio-isotopic tracers are particularly suitable. When the inhibitor has exactly the same composition and configuration as the determinant group of the antigen, the determination of antigen, antibody and inhibitor in mixtures containing all three is extremely difficult, and often impossible, by ordinary chemical methods. The analysis can readily be made, however, if either the antigen or the inhibitor contains a radioactive isotope; both may be labelled, with different radio-isotopes, but this is not usually necessary. In some of the experiments described here, for example, radioactive 3:5-diiodotyrosine and non-radioactive iodinated proteins were used. The possibility of exchange of ^{131}I between these two compounds in precipitin reactions carried out at about pH 7·5 can virtually be excluded, since exchange between iodide and either 3:5:diiodotyrosine or thyroxine is negligible at alkaline pH (Miller, Anderson, Madison & Salley, 1944; Frieden, Lipsett & Winzler, 1948).

When 3:5-diiodotyrosine is added, in amounts which only partially inhibit precipitation, to a mixture of iodinated protein antigen and its antiserum, the molecular ratio of antibody:antigen in the precipitate is usually less than that for the precipitate which separates in the absence of inhibitor. Woolf (1941) found that the addition of hapten effected a similar reduction in the antibody:antigen ratio of precipitates separating from azoprotein antigen-antibody systems. This is not surprising, since the action of the inhibitor can be attributed to its capacity to compete with the antigen for the reactive groups of the antibody. In effect, the inhibitor reduces the amount of available antibody in the solution, and with this change towards the excess antigen region there is a fall in the antibody:antigen ratio of the precipitates. When the con-

Table 3. *Composition of the unwashed precipitates separating from mixtures of iodinated proteins, their antibodies and 3:5-diiodotyrosine (inhibitor)*

Antigen: Iodinated human serum globulin.
Antiserum: Rabbit antiserum to horse serum globulin.
Inhibitor: 3:5-Diiodotyrosine (DIT) (containing ^{131}I).

(Experimental details are given in the text. All the amounts are given as mμ.mol./ml. of antiserum added.)

Antigen added (mμ.mol.)	DIT added (mμ.mol.)	Composition of precipitate: Antibody (mμ.mol.)	Antigen (mμ.mol.)	Antibody : antigen (Mol. ratio)	DIT: Total (mμ.mol.)	DIT: Free* (mμ.mol.)	DIT: 'Combined' (mμ.mol.)	'Combined' DIT : antibody (Mol. ratio)
7·03	0	12·55	3·85	3·3	0	0	0	—
	440	5·34	2·98	1·8	3·00	2·73	0·27	0·05
	1320	4·70	2·78	1·7	7·89	8·15	−0·26	0
	3520	3·44	2·17	1·6	15·10	15·04	0·06	0·017

* Determined by carrying out the precipitin reaction in a solution containing 5% D_2O, and measuring the D_2O content of the precipitate and supernatant solution.

centration of antigen is very high, however, we have found that the addition of even a very considerable excess of inhibitor does not lead to any significant change in the antibody:antigen ratio for the precipitate.

Competition between inhibitor and antigen apparently results in an equilibrium between flocculated antibody-antigen complexes and soluble complexes (antibody-inhibitor, and possibly, if antibody is bi- or multi-valent, antigen-antibody-inhibitor also). The flocculated particles might also contain inhibitor attached to some of the serologically active antibody groups, but no information on this point is apparently available in the literature. The results recorded here show that 'partially inhibited' precipitates obtained with the iodinated protein antigen-antibody system contain little or no inhibitor, and this observation appears to have an interesting bearing on antibody valence.

The antigen used in our experiments was undoubtedly multivalent, for it contained about sixty 3:5-diiodotyrosine residues per molecule, and furthermore it gave precipitates with antibody: antigen molecular ratios up to over 10:1 in the region of antibody excess in the absence of diiodotyrosine. If the antibody also is multivalent, and the precipitated complex has a 'lattice structure' (cf. Marrack, 1938) it seems highly probable that many of the antibody molecules in solution will have some of their valences 'blocked' by combination with diiodotyrosine molecules. Provided the remaining 'unblocked' valences are at least two in number, there seems no reason why these antibody-inhibitor complexes should not take part in lattice formation. If the antibody is bivalent and has only one valence blocked, or alternatively, if a few or even all but one of the valences of multivalent antibody are blocked, it seems reasonable to suppose that these antibody-inhibitor complexes should coprecipitate, as do Heidelberger's 'univalent or incomplete' antibodies (Heidelberger & Kendall, 1935; Heidelberger, Treffers & Mayer, 1940; cf. Kabat & Mayer, 1948). Thus with a multivalent antibody one might reasonably expect to find the specific inhibitor attached to the precipitates in these 'partially-inhibited' systems, as represented diagrammatically, for a bivalent antibody, in Fig. 2.

If any inhibitor is attached to the precipitated antibody, one might expect that the amount would be measurable, e.g. more than one molecule of inhibitor per twenty molecules of antibody, for it seems improbable that an insoluble antibody-antigen-inhibitor complex with a molecular ratio of, say, 20:11:1 would become soluble if one only of the antigen molecules were replaced by inhibitor. In most of our experiments with the 'partially inhibited' iodoprotein serological system the amount of inhibitor in the precipitate was significantly less than this. We feel justified, therefore, in suggesting that our observations support the view that the rabbit antibodies we have studied are univalent, i.e. each molecule has only one combining site for the diiodotyrosine group. On the basis of univalent antibodies, specific inhibition can readily be explained. Antigen and inhibitor compete for antibody, and where the antibody combining site is blocked by inhibitor, this antibody molecule is not able to precipitate, or be precipitated by, antigen.

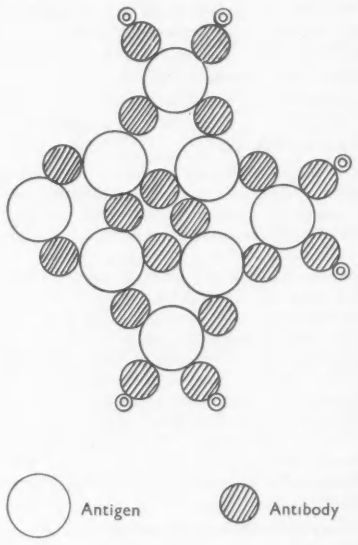

Fig. 2. Hypothetical lattice formed by iodinated protein antigen and bivalent antibody in the presence of the specific inhibitor (3:5-diiodotyrosine).

Several authors have obtained evidence that precipitating antibodies are univalent (see, for example, Boyd, 1942; Haurowitz & Schwerin, 1942). There is also evidence of 'incomplete', 'partial' or 'blocking' antibodies (see, for example, Heidelberger & Mayer, 1940; Race, 1944; Morgan & Schütze, 1946) which are generally described as 'univalent'. Since, however, these 'partial' antibodies do not precipitate or agglutinate the appropriate antigens they are not comparable with those present in the rabbit antisera we have studied. Further support for the theory of univalence of precipitating antibodies was recently obtained by Porter (1950) in an interesting new approach to serological inhibition. By enzymic hydrolysis of rabbit antiovalbumin Porter obtained a specific inhibitor which combined with, but did not precipitate, the antigen. Only one molecule of

inhibitor was obtained from each molecule of antibody, and Porter concludes that this result is more in keeping with the occlusion theory of precipitation (Boyd, 1942) than the lattice theory.

In these inhibition tests with hydrolysed antiovalbumin, Porter (1950) found that ovalbumin-antiovalbumin precipitates separating in the presence of the specific inhibitor (hydrolysed antibody) contained no inhibitor, and this is rather surprising if, as is generally believed, antigens such as ovalbumin are multivalent. This conclusion was based, however, on the results of total nitrogen determinations and the assumption that all the antigen and nearly all the antibody were precipitated, and it would be difficult to justify these assumptions. A complete analysis of these precipitates, for antigen, antibody and inhibitor, would provide very interesting information, and we suggest that isotopic labelling could profitably be applied to this serological system.

SUMMARY

1. Using ^{131}I-labelled antigen or inhibitor, a quantitative study has been made of the iodinated protein antigen-antibody precipitin reaction in the presence of the specific inhibitor, 3:5-diiodotyrosine.

2. The addition of inhibitor usually caused a fall in the antibody:antigen ratio of the precipitate, due apparently to the inhibitor reducing the concentration of free or effective antibody.

3. Some of these precipitin tests were carried out with mixtures containing 5% deuterium oxide. This made it possible for determinations to be made of the amount of supernatant solution, and hence the amount of free inhibitor, in the unwashed precipitates.

4. The amount of combined inhibitor in the precipitates was very small and sometimes immeasurable; at most, it was equivalent to one molecule of 3:5-diiodotyrosine per twenty molecules of antibody in the precipitate.

5. The results are discussed in relation to antibody valence, and it is suggested that they support the view that the precipitating antibodies studied are univalent.

We are very grateful to Dr G. Popják for having made some of the heavy water determinations, and we are indebted to the Medical Research Council and the Central Research Fund of the University of London for grants to one of us (A. W.) towards the cost of these investigations.

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The Occurrence of the Tricarboxylic Acid Cycle in Yeast

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Whereas the tricarboxylic acid cycle has been firmly established in mammalian tissues as a major pathway of tissue respiration, there is no unanimous concensus of opinion about its significance in micro-organisms (cf. Karlsson & Barker, 1948; Deffner & Franke, 1939; Clifton & Logan, 1939 on the one hand, and, for example, Oginsky, Smith & Umbreit, 1949; Baskett & Hinshelwood, 1950 on the other). Equally conflicting views are held with regard to the occurrence of a similar cycle in yeast. Citric acid formation in yeast has been observed by many workers, e.g. with whole cells in the presence of metallic ions (Wieland & Sonderhoff, 1932; Virtanen & Sundman, 1942) and in cell-free extracts fortified with adenosinetriphosphate (ATP), coenzyme A and high concentrations of Mg^{++} (Novelli & Lipmann, 1950). The oxidation of isotope-labelled acetate by yeast has been studied by Lynen (1947) and by Weinhouse & Millington (1947) who conclude that the tricarboxylic acid cycle is active in yeast.

On the other hand, Krebs (1949) believes that most of the earlier work is invalidated by the use of impure yeast. He points out further that the rates of citric and succinic (Kleinzeller, 1941) acid formations are slow when compared with the respiration, and he was unable to find evidence for the oxidation of citric and succinic acids by pure yeast. He believed that the non-oxidation of succinate, in particular, was not due to impermeability. The problem of the intermediary stages of substrate oxidation in yeast he therefore regarded as unsolved.

The present experiments arose from work upon the action of fluoroacetate on yeast, and were carried out in the hope of gaining both qualitative and quantitative information about the pathway of citric acid breakdown in enzyme preparations from pure yeast. Whilst intact cells do not metabolize added tricarboxylic acids, cell-free extracts can be obtained capable of metabolizing these acids at a rate corresponding to up to 40% of the rate of oxidation of pyruvate by the intact cells. α-Ketoglutaric acid has been identified as the end product of citrate breakdown provided that its further oxidation to oxaloacetate and pyruvate is inhibited by semicarbazide or cyanide. In contrast to the high sensitivity of animal α-keto-acid decarboxylases to arsenite and lewisite, high concentrations of these inhibitors did not affect α-ketoglutarate breakdown in cell-free yeast extracts. The results presented establish the tricarboxylic acid cycle as a significant pathway of substrate oxidation in the strains of *Saccharomyces cerevisiae* investigated.

METHODS AND MATERIALS

Reagents. Oxaloacetic, DL-*iso*citric, *cis*aconitic and α-ketoglutaric acids, as well as recrystallized sodium pyruvate, were prepared in this laboratory by Mr R. W. Wakelin. The pyridine nucleotides used in Exp. 2*B*, Table 1, were, respectively, a crude preparation from liver of coenzyme II (Co II) kindly provided by Dr Lotspeich and a sample of 60% pure coenzyme I (Co I) prepared in this laboratory. ATP was prepared from rabbit muscle by the method described by Umbreit, Burris & Stauffer (1945); its purity was 70%. Weights of ATP are expressed in mg. of Ba salt, but the latter was converted to the Na salt before use. Semicarbazide hydrochloride, KCN and sodium citrate.$2H_2O$ were British Drug Houses Ltd. reagents. All reagents were freshly made up and neutralized, if necessary, immediately before use. Phosphate buffers were mixtures of KH_2PO_4 and $Na_2HPO_4.12H_2O$.

Analytical methods. Citrate was determined by the method of Pucher, Sherman & Vickery (1936), as modified by Buffa & Peters (1950). Steam-volatile, non-keto-acids were estimated by the method of Long (1938); HCN did not interfere in the final titration if bromothymol blue was used as indicator. The method of Friedemann & Haugen (1943) was used for the estimation of keto-acids; final readings were carried out on a Beckman spectrophotometer at 420 and 540 mμ. A correction had to be applied in the presence of semicarbazide. The 2:4-dinitrophenylhydrazones of the keto-acids were separated chromatographically (Cavallini, Frontali & Toschi, 1949) using water-saturated *n*-butanol with 2% NH_4OH as suggested by Dr R. V. Coxon (private communication). Dicarboxylic acids were separated from the tricarboxylic acids and from each other by one-dimensional ascending paper partition chromatography using Whatman no. 4 filter paper and water-saturated *n*-butanol (88%), $CHCl_3$ (10%), 98% formic acid (2%) (all v/v). The chromatograms were developed with phenol red.

Preparation of extract. The source of the enzyme was freshly bought, commercial baker's yeast (Distillers Company Ltd.). Identical results were, however, obtained with a pure strain of *S. cerevisiae* grown in the laboratory at 25° on a liquid tryptic casein-marmite-glucose medium, pH 5·0. For the preparation of the extract the yeast was washed twice on the centrifuge and then suspended in ice-cold 0·9% KCl (1 : 3). Disintegration was carried out in the cold room in a Mickle shaker (a cup fastened at the end of an electromagnetically operated tuning fork) with the aid of small glass beads (Ballotini 14). The suspension was then centrifuged in

the cold for 20 min. at 6000 rev./min. (7000 *g*). The efficiency of cell distintegration could be roughly estimated from the stratified appearance of the sediment. It must be emphasized, however, that it was not found possible to standardize fully the extent of cell disintegration, and experiments with different preparations even from the same batch of yeast are thus not strictly comparable. The cell-free extract was dialysed if required in thin collodion tubes in a rocking dialyser in the refrigerator, against 0·1 M-phosphate, pH 7·1, or 1·5% KCl as stated. All solutions were made up in glass-distilled water.

Incubation and analysis. The extracts were incubated usually in 50 ml. Erlenmeyer flasks shaken in a Warburg bath. Conventional Warburg manometers were used for anaerobic experiments. Each flask contained 2 ml. of enzyme in a final volume of 3 ml. Volumes were adjusted with 0·9% KCl. At the conclusion of the incubation the flask contents were acidified with 1 ml. of freshly prepared 25% (w/v) trichloroacetic acid. The protein precipitate was spun off, the supernatant made up to volume and suitable portions taken for analysis. All values given in the tables represent the mean of determinations on at least two duplicate flasks with a deviation from the mean of no more than ±5%.

RESULTS

In Table 1 are summarized experiments illustrating the utilization of citrate by extracts of fresh commercial baker's yeast. It will be noted (Exp. 1) that ATP, in the presence of Mg^{++}, significantly accelerates the rate of citrate disappearance in the non-dialysed extract.

Exp. 2 shows that the marked effect of ATP + Mg^{++} consistently observed in the fresh extract is abolished on dialysis or on addition of cyanide. There always remains, however, a residual cyanide-stable system metabolizing citrate under these conditions at about two-thirds the rate in the fresh extract fortified with ATP and Mg^{++}. Once the cyanide-sensitive, ATP-requiring system has been inactivated by dialysis, it is not reactivated by the addition of Co I or Co II in the quantities shown in Exp. 2*B*. The system can be reactivated, on the other hand, by the addition of a boiled extract of starved yeast (shaken 4 hr. at 37°, then spun and washed, and thereupon extracted at 90° for 5 min.) equivalent to the volume of enzyme solution used (Exp. 3). These results are corrected for the citrate present in the boiled extract. The system active in the absence of ATP is very much more stable than the cyanide-sensitive, ATP-requiring system. Even after long periods of dialysis there is no evidence that the former needs any dialysable cofactors or inorganic phosphate (cf. Table 2).

As the absence of oxygen does not completely inhibit the activity of the fresh extract, there appears to be a coupled reduction with a reducible substrate; the latter is evidently not entirely removed by even prolonged periods of dialysis. The greater inhibition of citrate disappearance observed under anaerobic conditions than in the presence of cyanide may be accounted for by the finding, in separate experiments, that 0·01 M-cyanide inhibited the endogenous oxygen uptake of fresh yeast by only about 90%. This is in agreement with the results of Dixon & Elliott (1929).

No information could be obtained on the nature of the hydrogen acceptor in the cyanide-stable system. That it is not oxaloacetate is shown by Exp. 2, Table 2, in which the keto-acid fixative cyanide did not interfere with the anaerobic citrate dissimilation; nor could any traces of malate, fumarate or succinate be discovered by chromatography. Had there been a direct split of citrate to oxaloacetate and acetate, as suggested by Deffner & Franke (1939), there

Table 1. *Citrate utilization by cell-free yeast extract*

(Each flask contained 10 μmol. citrate, 0·08 M-phosphate buffer, pH 7·1, ATP and Mg^{++} added as shown in quantities of 1 mg. and 100 μg., respectively. Flasks incubated at 37° for 30 min. Gas phase: air.)

Exp. no.	Extract	Additions	Citrate metabolized (μmol.)
1	Non-dialysed	—	5·6
		ATP	6·8
		ATP + Mg	9·1
2*A*	Non-dialysed	—	6·6
		0·01 M-KCN	5·7
		ATP + Mg	9·1
		ATP + Mg + 0·01 M-KCN	6·8
	Boiled	0·01 M-KCN	0
2*B*	Dialysed 5 hr. against phosphate	—	5·7
		ATP + Mg	6·2
		ATP + Mg + Co II (50 μg.)	6·2
		ATP + Mg + Co I (100 μg.)	6·1
3	Dialysed 4 hr. against phosphate	—	7·9
		ATP + Mg	8·3
		ATP + Mg + boiled yeast juice containing 3·2 μmol. citrate	12·4

Table 2. *Citrate utilization in dialysed extract*

(Each flask contained 0·03 M-phosphate pH 7·1 or 0·02 M-veronal acetate pH 7·1 as indicated, and 10 μmol. citrate. The N_2 was free from O_2. Cyanide was used in a concentration of 0·01 M. The flasks were incubated for 30 min. at 37°.)

Exp. no.	Age of extract (hr.)	Dialysis*	Gas phase	Additions	Citrate metabolized (μmol.)
1	0	−	Air	Phosphate	6·0
		−	N_2	Phosphate	3·3
		−	Air	Phosphate-KCN	5·0
2	5	+	N_2	Phosphate-KCN	3·2
3	12	−	Air	Phosphate	2·2
		+	Air	Phosphate	1·6
		+	Air	Phosphate-KCN	0·9
		+	N_2	Phosphate	0·9
		+	Air	Veronal	1·8
4	24	+	Air	Phosphate	0·7
		+	Air	Veronal	0·9
		+	N_2	Phosphate	0

* Dialysis was against KCl and its duration corresponds to the age of the extract.

Table 3. *Formation of keto-acids from citric acid in dialysed extracts*

(Each flask contained 0·08 M-phosphate, pH 7·1. Temp. 37°. Duration 30 min. Gas phase: air. Dialysis against 1·5% KCl, for 4 hr.)

Exp. no.	Additions	Initial concn. of citrate (μmol.)	Citrate metabolized (μmol.)	Keto-acid formed (μmol.)	$\frac{E_{420}}{E_{540}}$*	Remarks
1	—	—	—	0·9	1·53	Preparation sterile
	Citrate	6·0	3·1	2·6	1·71	
	Citrate + 0·5 mM-As_2O_3	6·0	2·6	2·7	1·64	
	Citrate + 0·1 M-semicarbazide	6·0	3·2	2·9	1·83	
2	0·01 M-Semicarbazide	—	—	0·3	1·86	—
	Citrate	3·0	2·1	—	1·68	—
	Citrate + 0·01 M-semicarbazide	3·0	2·1	1·8	1·90	Identified as α-ketoglutarate on chromatogram
3	0·01 M-KCN	—	—	0·7	1·53	Cyanhydrins split by 5 min. boiling of acidified solution
	Citrate + 0·01 M-KCN	5·0	2·0	2·3	1·74	
	α-Ketoglutarate + 0·01 M-KCN (boiled enzyme)	—	—	—	1·83	

	E_{420}/E_{540}
* Pure α-ketoglutarate	1·90
Pure pyruvate	1·31

should also have occurred a formation of volatile acid; no trace, however, could be discovered in large-scale experiments on steam distillation of dialysed extracts which had metabolized 40–60 μmol. of citric acid in the presence of 0·01 M-cyanide.

Owing to the large concentration of keto-acid precursors, both in the undialysed extract and in the boiled yeast juice, attempts to estimate the keto-acid formed as a result of citrate utilization by the ATP-activated system did not yield significant results. Keto-acid roughly equivalent to the citrate metabolized was indeed formed. It was greatly diluted, however, with the pyruvate appearing even in the control, in the absence of citrate. It was not possible, therefore, to estimate accurately the E_{420}/E_{540} ratio of the hydrazone of the keto-acid formed from citrate. If, however, citrate disappearance was studied in the well-dialysed extract in the presence of various keto-acid reagents, yields of α-ketoglutaric acid approaching the theoretical could be obtained (Table 3). Parallel determinations of oxygen uptakes in extracts whose blank respiration was negligible did not lead to consistent results.

The results of Exp. 1, Table 3, suggested that the further breakdown of α-ketoglutarate was not inhibited in this case by arsenite. Other experiments on the effect of arsenicals are illustrated in Table 4. It will be noted that neither arsenite nor lewisite oxide inhibit the breakdown of α-ketoglutarate in the yeast system. This result is in strong contrast to the sensitivity of the enzyme from mammalian tissue to arsenite and to lewisite (Peters, 1950). It may here be noted that yeast carboxylase is also insensitive to arsenite (Neuberg & Rosenthal, 1913).

Table 4. *Breakdown of α-ketoglutarate*

(Each flask contained 0·07 M-phosphate, pH 7·1, 5 μmol. α-ketoglutarate. Duration of dialysis: 5 hr. Incubated at 37° for 30 min. Gas phase: air.)

Exp. no.	Treatment	Additions	Total keto-acid remaining (μmol.)	E_{420}/E_{540}	α-Keto-glutarate metabolized (μmol.)
1	Dialysed against 1·5% KCl	—	1·9	1·63	4·1
		10 μg. Cocarboxylase	1·8	1·63	4·0
		1·0 mM-As_2O_3	2·2	1·70	3·5
		Acidified control	5·0	1·96	—
2	Dialysed against 1·5% KCl	—	2·7	1·81	2·9
		0·3 mM-Lewisite oxide	2·6	1·80	3·1
	Dialysed against 0·1 M-Na_2HPO_4, then neutralized to pH 7·1	—	5·0	1·58	2·9
		20 μg. Cocarboxylase	4·9	1·56	3·1

Table 5. *Aconitase and* isocitric *dehydrogenase*

(Each flask contained 0·08 M-phosphate, pH 7·1. 0·01 M-Semicarbazide. Enzyme dialysed 5 hr. against 1·5% KCl. Incubated at 37° for 30 min. Gas phase: air.)

Exp. no.	Additions	Citrate remaining (μmol.)	Tricarboxylic acid metabolized (μmol.)	Keto-acid formed (μmol.)	E_{420}/E_{540}
1	—	—	—	0·4	1·70
	DL-*iso*Citrate (20 μmol.)	—	—	2·4	1·81
	*iso*Citrate + 0·005 M-iodoacetate	—	—	2·4	1·95
2	—	—	—	0·5	1·62
	Citrate (5 μmol.)	2·0	3·0	2·0	1·72
	cis-Aconitate (5 μmol.)	2·5	2·5	2·0	1·69

Table 6. *Citrate utilization by extract from pure yeast*

(Each flask contained 0·08 M-phosphate, pH 7·1. Incubated at 37° for 30 min. Gas phase: air.)

Extract	Additions	Citrate metabolized (μmol.)	Keto-acid formed (μmol.)	E_{420}/E_{540}
Freshly prepared	Citrate (8 μmol.) + ATP (1 mg.) + Mg^{++} (100 μg.)	7·3	—	—
Dialysed against 1·5% KCl for 4 hr.	0·01 M-Semicarbazide	—	1·1	1·48
	Citrate (8 μmol.) + 0·01 M-semicarbazide	3·2	3·9	1·80

Addition of cocarboxylase to the normal extract dialysed against potassium chloride had no effect. This is not surprising as yeast carboxylase, for instance, is only split at alkaline pH; the apoprotein is well known to be very unstable. Dialysis against alkaline phosphate did indeed irreversibly inactivate the further breakdown of pyruvate, without affecting the oxidation of α-ketoglutarate to oxaloacetate and pyruvate, as judged by the values of E_{420}/E_{540} shown in Table 4.

These experiments make it clear that the cyanide-stable system present in the well-dialysed extract can convert citrate to pyruvate via α-ketoglutarate. This implies the presence of aconitase, and the extract was in fact found, when tested on *cis*-aconitate or *iso*citrate, to contain sufficient enzyme to establish the aconitase equilibrium (about 90% citric acid) in less than 30 sec. under experimental conditions. Table 5 shows in agreement with this that the behaviour of *iso*citric and *cis*aconitic acids is identical with that of citric acid. The *iso*citric dehydrogenase of yeast was not inhibited even by high concentrations of iodoacetate in the 30 min. period of the experiment. The enzyme thus appears to differ from the iodoacetate-sensitive *iso*citric dehydrogenase described by Breusch & Keskin (1943–5) in pigeon-breast muscle preparations.

The clear demonstration of the significant stages in the tricarboxylic acid cycle in the cell-free extract from fresh baker's yeast is in contrast to the findings of Krebs (1949) with pure yeast. To eliminate any possible effect of contamination in the starting material, the essential points were confirmed upon three occasions with pure cultures of *S. cerevisiae* grown in the laboratory: Table 6 shows the results of one such experiment.

DISCUSSION

The results presented in this paper unequivocally establish the occurrence in yeast cells of various reactions of a cycle related to the tricarboxylic acid cycle as present in mammalian cells. Under optimum conditions a $q_{cit.}$ (μl. citrate metabolized/hr./g. fresh weight at 37°) of up to -1000 has been obtained in the extract. The q_{O_2} of fresh yeast in the presence of excess pyruvate (μl. oxygen/hr./g. fresh weight) in phosphate buffer at pH 6·2 was of the order of 5–7000. This value has been corrected for the high endogenous oxygen uptake on the assumption that added substrate does not significantly suppress the latter. Even if the true correction should be smaller (cf. Winzler, 1940), it is obvious that the rate of citrate utilization in the extract ($q_{cit.} - 1000$) could account for a significant portion of the $q_{pyr.}$ of the intact cell (μl. pyruvate/hr./g. fresh weight = about -2500 on the assumption that the pyruvate is completely oxidized). It must be mentioned that, while the results of Novelli & Lipmann (1950) on the citrate synthesis in the cell-free extract could be confirmed, the rate of this synthesis ($q_{cit.} + 50$) in extracts from pantothenate-enriched yeast in the presence of high concentrations of magnesium was considerably lower than the rate of citrate utilization in the absence of excess magnesium. There was, however, no appreciable acetate utilization or increased oxygen uptake when a freshly prepared extract from a pantothenate-enriched yeast was incubated with acetate in the presence of ATP and cysteine. On the other hand, in the course of another research now in progress in extension of work on kidney by Liébecq & Peters (1949), intact yeast cells, when incubated at 37° in the presence of fumarate and fluoroacetate at pH 6·1, were observed after an initial lag phase to accumulate citrate at a rate ($q_{cit.}$ about $+700$) comparable to the rate of citrate utilization by the extract. The failure of the cell-free extract to activate acetate to any large extent does not, therefore, invalidate the view that the cycle constitutes a major pathway of oxidation in the intact cell. It is, indeed, not known whether the experimental conditions in the extract represent the optimum conditions for citrate metabolism. The pH value of 7·1 used throughout is more alkaline than the overall internal pH of the yeast cell as reported by Conway & O'Malley (1946). Repetition of citrate-utilization and α-ketoglutarate-formation experiments at pH 6 gave similar results to the experiments carried out at a more alkaline pH, but at a somewhat slower rate (about 60 %).

It is assumed in this discussion that the ATP-requiring, cyanide-sensitive part of citrate breakdown also leads to α-ketoglutaric acid. That this could not be proved has already been pointed out in the experimental section. Even if this assumption were not correct, however, it would not upset the argument that the tricarboxylic acid cycle plays a significant part in the oxidative metabolism of the cell, as a large percentage of the citrate breakdown undoubtedly leads to α-ketoglutaric acid. In this connexion it may be interesting to refer to the recent finding of Kornberg (1950) of two separate *iso*citric dehydrogenases in yeast both leading to α-ketoglutaric acid. They may be related to the existence of the two different pathways of citrate breakdown described in this paper.

Several other possible pathways of citrate breakdown in the dialysed extract may be excluded: chromatography did not reveal any formation of C_4-dicarboxylic acids, nor were any steam-volatile acids formed if the reaction was stopped at the α-ketoglutarate level. Acetonedicarboxylic acid, a possible intermediate of citrate breakdown (Challenger, Subramaniam & Walker, 1927), can also be excluded: it does not give pentabromoacetone under the conditions of the citrate estimation as it is rapidly destroyed by permanganate; its 2:4-dinitrophenylhydrazone has a E_{420}/E_{540} ratio of 1·86, i.e. similar to that of α-ketoglutarate, but it is estimated to the extent of only 7 % under the conditions of the Friedemann-Haugen α-keto-acid estimation. That the enzymes of the cycle operating in yeast differ from those in mammalian cells is shown by the insensitivity of α-ketoglutarate oxidation to arsenicals. The enzyme from animal tissues (Stumpf, Zarudnaya & Green, 1947) has been reported to be an aneurinpyrophosphate protein more easily dissociated, at least in acid solution, than pyruvic oxidase. The present results provide no evidence that cocarboxylase is required for the decarboxylation of α-ketoglutarate in yeast.

The existence in yeast of mitochondria containing oxidative enzymes has been shown by Chantrenne (1943–5). These are not sedimentable at the centrifugal force (7000 ***g***) employed in the preparation of the extract (cf. also Nyman & Chargaff, 1949). They differ thus from the cyclophorase-containing mitochondria of kidney homogenates.

SUMMARY

1. A cell-free extract of baker's yeast rapidly metabolizes citrate. For maximum activity in the fresh non-dialysed extract adenosinetriphosphate and magnesium are required.

2. Even after thorough dialysis there remains a citrate-metabolizing system which is not affected by cyanide. Adenosinetriphosphate and magnesium are now without effect unless boiled yeast juice is also added.

3. Yields of α-ketoglutarate approaching the theoretical are obtained from citrate in the dialysed extract. The further breakdown of α-ketoglutarate to oxaloacetate and pyruvate is not inhibited by arsenicals.

4. As similar results could be obtained with a pure sample of *Saccharomyces cerevisiae* grown in the laboratory it is concluded that the tricarboxylic acid cycle represents a significant pathway of substrate oxidation in yeast.

I should like to thank Prof. R. A. Peters, F.R.S., for his encouragement and advice. Dr D. D. Woods kindly provided the sample of pure yeast. The help of Dr L. Stocken in the preparation and estimation of adenosinetriphosphate is gratefully acknowledged.

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Note on an Improved Method for the Preparation of Oxythiamine

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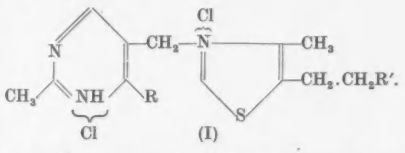
Oxythiamine (I; R=R′=OH) is of some interest as an antagonist of vitamin B_1 (Soodak & Cerecedo, 1944; Eusebi & Cerecedo, 1949; Daniel & Norris, 1949), more especially since it has been reported to confer significant protection in mice against the Lansing strain of the poliomyelitis virus (Jones, Foster & Henle, 1948).



(I)

It was first prepared synthetically by Bergel & Todd (1937), but this procedure is laborious and a simple method of preparation from commercially available aneurin (thiamine, I; R=NH_2; R′=OH) is clearly desirable. Soodak & Cerecedo (1944) converted aneurin into oxythiamine in 50–70% yield by treatment with nitrous acid, but this method did not prove very satisfactory in our hands. 4-Amino-pyrimidines are generally readily converted into the 4-hydroxy compounds by boiling with dilute acids and this seemed to offer an attractive simple route to oxythiamine; it was already known (Buchman & Williams, 1935; cf. Barger, Bergel & Todd, 1935) that treatment of aneurin with concentrated hydrochloric acid in a sealed tube at 150° gave chloro-oxythiamine (I; R=OH; R′=Cl), the side-chain hydroxyl having been replaced by chlorine and the 4-amino group by hydroxyl. Milder conditions were

accordingly tried, the reaction being followed qualitatively by the thiochrome test, and it was found that consistently good yields of oxythiamine, substantially free from aneurin, could be obtained by simply refluxing aneurin with 5N-hydrochloric acid for 6 hr.; shorter periods of refluxing result in incomplete conversion and the use of stronger acid in the formation of some chloro-oxythiamine.

EXPERIMENTAL

Aneurin chloride hydrochloride (2 g.) was refluxed for 6 hr. with conc. HCl (50 ml.) and water (50 ml.). The product was evaporated to dryness under reduced pressure and the residue redissolved and re-evaporated with successive 50 ml. batches of ethanol and methanol. The final residue was dissolved in warm methanol (50 ml.); dry ether (75 ml.) was added and the mixture set aside to crystallize, finally at 0°. Oxythiamine chloride hydrochloride (I; R=R′=OH) (1·60 g.; 80%) separated in rosettes of flattened needles, m.p. 195° (decomp.), which were filtered off, washed with ether and dried in a vacuum desiccator. (Found: N, 12·0, 12·3; Cl, 21·3, 21·1, 20·8%; equiv., by electrometric titration, 332. Calc. for $C_{12}H_{17}O_2N_3SCl_2$: N, 12·4; Cl, 21·0%); oxythiamine chloride is a very weak base (pK_a less than 1) and the hydrochloride slowly loses HCl on drying *in vacuo*. The light absorption curve shows maxima at 2680, 2600, 2280 and 2210 A. (ϵ=9100, 9100, 10,100, 10,100) in alkaline (M/15-Na_2HPO_4) and at 2650, 2580, 2280 and 2230 A. (ϵ=10,800, 9300, 9500, 7900) in acid (0·1N-HCl) solution. Dr H. E. Cox kindly assayed two preparations for aneurin and found 0·00015% and less than 0·0001% respectively (Soodak & Cerecedo, 1944, report 0·1–0·4% for material prepared by their method). The dipicrate crystallizes from water in thin prismatic needles, m.p. 111° (Soodak & Cerecedo, 1949, report m.p. 102–108°). (Found: C, 39·5, 39·5; H, 3·6, 3·6. Calc. for $C_{24}H_{21}O_{16}N_9S$: C, 39·9; H, 2·9%.

SUMMARY

Oxythiamine, essentially free from aneurin, can be prepared in 80% yield by refluxing aneurin with 5N-hydrochloric acid for 6 hr.

I am much indebted to Dr H. E. Cox for the aneurin assays and to Dr E. A. Braude for the light-absorption data.

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